2015

EFFECTS OF PERFLUORINATED COMPOUNDS (PFCs) ON METABOLIC TISSUES AND THE BENEFITS OF CALORIC RESTRICTION

Deanna M. Salter
University of Rhode Island, deanna_salter@my.uri.edu

EFFECTS OF PERFLUORINATED COMPOUNDS (PFCs) ON METABOLIC TISSUES AND THE BENEFITS OF CALORIC RESTRICTION

BY

DEANNA M. SALTER

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOMEDICAL AND PHARMACEUTICAL SCIENCES SPECIALIZING IN

PHARMACOLOGY AND TOXICOLOGY

UNIVERSITY OF RHODE ISLAND

2015

DOCTOR OF PHILOSOPHY IN BIOMEDICAL AND PHARMACEUTICAL SCIENCES SPECIALIZING IN PHARMACOLOGY AND TOXICOLOGY

OF

DEANNA M. SALTER

APPROVED:

Dissertation Committee:

Major Professor Angela L. Slitt

Ruitang Deng

Matthew Delmonico

Nasser H. Zawia

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND
2015

ABSTRACT

The CDC states that there has been a dramatic increase in obesity from 1990 to 2010. Type-II diabetes and obesity prevalence are increasing worldwide. Often, obesity and Type-II diabetes are concurrent, and predispose individuals to development of fatty liver disease, referred to as Non-alcoholic fatty liver disease (NAFLD). Perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) are two commonly studied perfluorinated compounds (PFC's) that are considered environmental toxicants that have the potential to elicit diabetic and NAFLD phenotypes. This dissertation presents novel findings of gaps within the literature to date. Traditionally, diabetes and obesity have been discussed in regard with genetics, diet and old age. Now, risk factors that also need to be considered are environmental chemicals. We found that PFOS elicits an insulin-resistant phenotype in adult mice, where they were not utilizing glucose as readily compared to the controls. The effect of PFOS on therapeutic management interventions has not previously been looked at. Here, we show that PFOS interferes with the Metformin-induced decrease in glucose. We also found a vast increase in the hepatic triglycerides with PFOS exposure. In this thesis, PFOS was administered in a sub-chronic low dose (100μg/kg) daily to mice fed *ad libitum* or placed on caloric restriction (CR) for five weeks. In the cohort we generated, we observed that PFOS exposure increased hepatic lipid content in mature male mice fed *ad libitum* and dampened the observed CR-induced decrease in hepatic lipids. PFOS administration did not affect glucose tolerance in *ad libitum* fed mice, but did interfere with CR-induced improvement of glucose tolerance. This was further associated with suppression of IRS-1 mRNA expression in liver. As hepatic lipid

content is closely tied to insulin and glucagon signaling for hepatic glucose production, it was determined whether the observed effects *in vivo* were due to PFOS-stimulated hepatic glucose production. Using cultured mouse hepatocytes under low glucose conditions, it was evaluated whether PFOS could enhance glucagon-stimulated glucose production. PFOS stimulated hepatocyte glucose production and also enhanced glucagon-induced glucose production. Furthermore, in HEPG2 cells, PFOS exposure (25 and 250 μM) significantly increased glucose output and AiCar, which suppresses glucose production, was ineffective in the presence of PFOS. These findings provide a mechanistic explanation for the decreased glucose tolerance in our *in vivo* mouse cohort. PFOS increases glucose output from the *in vitro* models, even when challenged with metformin or AiCar, as well as, decreases the glucose utilization in the *in vivo* mouse study suggesting that it has a glucagon-like effect.

PFOA is a perfluorinated carboxylic acid also commonly found in the environment. According to the EPA, low levels of PFOA are widely distributed in environmental media (Houde *et al.,* 2011; Gewertz *et al.,* 2013) and in the blood of the general United States population. The EPA states that PFOA is a known liver toxicant, development toxicant, and carcinogen in rodents. It has been detected in human liver, kidney and adipose tissue ranging from 0.3 to 3.8ng/g with the highest concentrations within the liver (Maestri *et al.,* 2006). In another study, PFOA is considered an obesogen to mid-aged mice where insulin and leptin levels were altered at a very low concentration (Hines *et al.*, 2009). PFOA is a Potent activator of peroxisome-proliferator activated receptor-alpha (Ppar-α) contributing to oxidative stress and activation of fatty acid oxidation pathways in hepatocytes. Given its persistence, the

purpose of this study was to evaluate whether PFOA treatment affects fatty acid oxidation, lipid synthesis, and antioxidant response gene expression in adipose tissue in adult male mice. Utilizing an exposure paradigm characterized by the Environmental Protection Agency, adult male mice were treated with 1.0 or 3.0 mg PFOA ammonium salt/kg for 7 days. Adipose tissue was collected and total RNA was isolated. Analysis of mRNA was completed by quantitative PCR. For the most part, gene expression in adipose tissue from vehicle- and PFOA-treated mice was similar. Literature lacks data on PFOA in adipose tissue and in human health, which continue to be discovered. Given its persistence, longer exposure periods and protein expression changes should be examined.

ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Angela L. Slitt, for giving me this opportunity to perform such meritorious research. Dr. Slitt has always helped to organize my thoughts and formulate cohesive presentations and papers that flowed smoothly. She has willingly written many letters of recommendations for me throughout the years and supported me through my research here at the University of Rhode Island. She has mentored me through this five-year journey and taught me how to be the best scientist I can be and for that, I am thankful.

I would like to thank my committee member, Dr. Ruitang Deng, for his input throughout each seminar and committee meeting, always helping me think outside the box into deeper research.

I would like to thank my committee member, Dr. Matthew Delmonico, for continually asking questions throughout committee meetings that truly engaged my mind to think about and how our work relates to human research. I would also like to thank him for meeting with me, boosting confidence in myself before major presentations. He is always willing to help in any way that he can and is always readily available to meet, and for that, I am extremely appreciative.

I would like to thank my committee member, Dr. Bongsup Cho, for his encouraging words throughout the five years of being in this program. I always loved speaking the little Korean that I knew to Dr. Cho and him responding so kindly to it. He always knew how to uplift and motivate me to be better and work hard each and everyday.

I would like to thank my committee member and also chair, Dr. Brenton DeBoef, for comedic relief in each committee meeting by asking me to speak to the audience as if I am speaking to a fourth grader about my research. It is always stressful being in front of a group of very intelligent PhD's in various fields, but somehow he always found a way to alleviate that stress.

I would like to thank the Biomedical and Pharmaceutical Sciences Chair, Dr. Bingfang Yan, for the motivational morning walks and talks. It was always a pleasure seeing someone else in the building in the early mornings from day to day. He would come in a talk to me about anything from research to personal life, and for that, I will always be thankful. He always believed in me and it came through when he would bring prospective professors or visiting speakers through our lab and always referred to me as one of the best students at URI when passing through. He had a peculiar way of motivating me by telling me to work harder and comparing my work ethic with his own and showing me his dissertation, telling me that I am not working hard enough, jokingly of course. From day one, he is one person that has influenced me and propelled me to move forward from the classroom to research, always leaving a positive vibe.

I would like to thank the Chemistry Department Chair, Dr. William Euler, who has provided opportunities to receive a teacher assistantship with the Chemistry Department in teaching General Chemistry and Organic Chemistry labs. He will never know how truly thankful I am for these opportunities, for I would not be able to be here without the support of the Chemistry Department.

I would like to thank my undergraduate advisor, Dr. Steven B. Symington, who has been a huge supporter of me since 2008. From his hard exams to writing grants in his Neuroscience class, he always pushed me to be the best student and scientist I could be. He was the first person who gave me a chance to perform research in the undergraduate research program of Rhode Island IDeA Network for Excellence in Biomedical Research (INBRE). The one email that changed my life in the best possible way was when I was assigned to him to perform research in his laboratory. It was also one of the happiest days of my life. Dr. Symington is a man who is extremely easygoing when it comes to golf and non-academic activities, however, when it came to class work and research, he was one of the toughest professors I can remember and I am extremely thankful for those few times he made me cry during presentations. He always made students stronger with his comments and his fantastic ways of teaching. He made sure I was fully prepared for graduate school and took me from being a terrified and nervous public speaker to a confident and well-rounded young scientist. Dr. Symington and I have had extremely inspiring discussions and he has always been, and will always be, a truly great mentor. Dr. Symington and the Salve Regina University Biology and Chemistry labs were truly the reason my love for research grew so rapidly. I thank you, Dr. Symington, from the terrifying Summer Friday mornings when I gave my presentations to the crazy talks and intellectual discussions over the past seven years; you are truly an inspiration.

I would not have been able to complete my graduate studies without the constant support of Marcia Wood Salter and Dr. Wilbur Mitchell Salter II. Mom and dad, you have been there for me since day one and always showed full support of whatever I

wanted to do. I cannot thank you enough for the late night calls after studying just to keep me motivated to keep going and always being there whenever I need you whether it be to come visit or to send care packages. The past few years have not been the easiest and I am so proud of you for being the strongest two people and dealing with so many difficult obstacles of life. Mom, you are truly my best friend and I thank you for everything you have done from driving to me in the middle of the night when I needed you to always being my outlet when school got tough. The support you have showed me and continue to show me is limitless. I cannot thank you enough for putting up with all the science talk that I brought you to for SOT in Phoenix, Arizona and to Dr. Symington's backyard pumpkin carving party! You have taught me so much that I will forever be thankful for and continue to be the best mother a daughter could ask for with the most positive attitude given any situation. You are the most caring person and the most phenomenal Registered Nurse the world has seen, thank you. Dad, you are and will forever be the smartest man I have ever known. I never take our talks for granted and always remember what you say. Your input to anything I do means so much to me and your words will forever positively impact my life. From performing surgery and being the best Veterinarian for years in the greater Boston area to cooking the best meals and growing the most amazing roses, there is nothing you can't conquer. I have learned so much from watching you as I've grown up and continue to learn from you everyday. As I've grown up, you and mom have taught me that nothing is impossible. You two have overcome some of the hardest obstacles together and are undoubtedly my heroes. I never would have been able to

reach this point in my life without you; my number one fans forever and always, thank you.

I would like to thank my grandmother, Constance Wood Lindquist, for helping support me through my years of studying. Her walks and talks are filled with so much family history and never get old. The days on the farm in Maine were a huge outlet when I needed a break from studying taking walks down memory lane with her and my mother. The three of us are the three best friends anyone could have; we can never stop laughing. We always have the best times together. In 2009, She had a timeshare in Sanibel Island, Florida where when asked what she wanted to do that day, she responded with getting tattoos. My mother and I looked at each other in disbelief. Sure enough, at 80-years of age, it's what she wanted to do and we all got tattoos! It was an experience I will never forget during a very hard time in my studies. Her and my mother even came to a seminar that I was giving for school. Thank you, Grandmama, for everything.

I would like to thank my three older brothers, Adam Lawrence Salter, Andrew Mitchell Salter and Daniel Justin Salter. They have all taught me life lessons that I still live by to this day. Adam, I know it has not been the easiest track for you, but watching you grow through this part of life that has been so challenging for you, is one of the most inspiring stories I will forever hold to my heart. Your determination and persistent willpower has been a huge factor in my graduate career. About eight years ago, I found out about your disease, which has overtaken your life and has inhibited you from many of life's natural activities such as college or a career. You always had high hopes of becoming a very wealthy person and have always been extremely smart

and I truly believe you will be hugely successful in the future. You are doing so well in your recovery and the past eight years, even though they have been undoubtedly difficult for all of us, you have continued to show us that you are not willing to give up. Your persistence to excel in life is nothing short of amazing. You have been an inspiration and continue to amaze me everyday; I love you and don't give up. Andrew, ever since I could walk, I always tried to keep up with you. Your athletic ability (which was and still is astounding!) and intelligence were things I longed for early in our childhood. As far back as I can remember you were the person I always aspired to be like and to this day, show me how great of a person you are. You have not only helped me get through tough situations, but you have motivated many others by teaching English in South Korea and helping athletes eat correctly and give motivational speeches to help them keep on track. I have always looked up to you as my mentor and I still wish I had your ability to speak in public. Your generosity and focus has been implanted in my life throughout graduate school and has helped sculpt me into a better person within the society. Thank you for being a wonderful mentor throughout my life, I love you. Dan, even though we have spent years apart, you are a huge part of my memories growing up, helping me and being there for me in any situation. You taught me how to be tough and to be a strong woman. In our recent visit, it made me extremely happy to see the man you have become, independent with your own business and a beautiful family. You molded me at an early age to be respectful and open-minded, always helping me reach for things that seem to be unreachable. Watching you and your success in life has been a huge part of my

inspiration through graduate school to make my own success. Thank you for your kindness and motivation, I love you.

I would like to thank my boyfriend, David Raymond Cyr, for the endless support and encouragement he gives on a daily basis. Since I met him, he is in lab on weekends when I need to be there, no question asked. If I have to be in lab while my parents need help with something, he is always the first to drive 3 hours to help them even when I can't. He has made my life exponentially easier and more efficient by helping with the little everyday tasks that graduate students don't usually have the time to do. Although I met him towards the end of my graduate school journey, the timing could not be more perfect when I needed someone like him to come into my life. He has made it so much better in every aspect. His words of encouragement and sincerity made the long nights and early mornings of graduate school bearable. He was always my go-to person when I needed to get away from school if even for just a phone call and he would talk as long as I needed to. I am truly grateful for having met him the time that I did.

I would like to thank my best friend, Jessie Barowski, for the countless texts and phone calls we have had over the past six years. Since being roommates at Salve Regina during the summer research session, we have been able to talk about anything. She has heard my ups and downs of graduate school and given me advice in how to advance forward with every obstacle I have encountered.

I would like to thank my former and present lab mates for helping with protocols and procedures in lab. I thank Supriya Kulkarni, Ajay Donepudi, Vijay More and Laura Armstrong for helping with the necropsy of my mouse study. I thank Pragati

Nahar, Prajakta Shimpi and Laura Armstrong for support in the laboratory for the past few years that we have been lab mates and I hope you all the best.

I would like the thank Ruchi Verma for her continuous support for the past couple of years of my graduate career. She would always be there whether it was about my studies or personal life. Whenever I forgot a lunch or did not have time to eat, she would always make me take the time to sit down and eat whatever she made for the day to ensure I was getting proper nutrients. She was always looking out for me and helping through daily obstacles. We have had the best and worst of times when it came to funding and school-related situations, and I am so grateful to have had her in the same building to talk to through these years. We have conquered so much together and I hope you the very best in finishing your graduate career.

Lastly, I would like to thank Ian Lester for putting up with all of the technical difficulties from the old to the new building. He has been to the rescue in many presentation and seminar talks from students to faculty. He does the entire behind the scenes work that needs to be recognized because we wouldn't be able to operate properly without computers when they get viruses or the help of IT when things go wrong. I cannot thank him enough for lending me chargers and adapters when I needed them at the last minute, not to mention the amount of keycards he has granted me after misplacing them. I am extremely appreciative of him printing our posters for the 2015 SOT conference. Ian, thank you very much for all that you have helped with along the way to make this journey a successful one.

DEDICATION

To my parents: my stability, strength, and support. It is to them that I dedicate the following dissertation, for their endless support, motivation and encouragement.

Marcia Wood Salter

Wilbur Mitchell Salter II

PREFACE

The following thesis project is in Manuscript Format. Based on our previous lab findings, the initial goals of this thesis project were to evaluate different models for changes and regulation of Nrf2, a transcription factor. However, after interesting observations with perfluorinated compounds (PFC's), my thesis evolved into a more focused and mechanistic study on Perfluorooctanesulfonic acid (PFOS), a major PFC. The bulk of the thesis work has been to understand whether PFOS exposure can interfere with the benefits of caloric restriction *in vivo* or in *vitro*. Epidemiology studies associating PFOS exposure with altered metabolics has further provided justification for studying PFOS effects.

Because the thesis is manuscript format, there have been experiments not included in the manuscript, but still essential to key observations of the thesis work. These have been included. Appendix material will also present findings for PFOS and PFOA with regard to adipose tissue and gene expression, along with work performed regarding the Nrf2 activating compounds, oleanolic acid (OA) and butylated hydroxyanisole (BHA), on interaction with a lithogenic diet that I generated early in my graduate career.

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Non-alcoholic fatty liver disease (NAFLD) statistics and etiology

NAFLD is a common chronic liver disease that is increasingly growing within the general population affecting 30% of adults, 20% of children and 69% of type 2 diabetic (T2D) patients in the United States (Ahmed *et al.,* 2015; Leite *et al*., 2009; McCullough, 2006). Features of metabolic syndrome are commonly found in NAFLD such as obesity (67-71%), disturbed lipid profiles (57-68%) and hypertensive (36-70%) (Ahmed *et al.*, 2015). NAFLD is defined by evidence of steatosis by imaging or histology without any secondary hepatic fat accumulation such as alcoholic consumption (Chalasani *et al.*, 2012). Major risk factors for NAFLD include obesity, type-2 diabetes (T2D) and dyslipidemia (Chalasani *et al.*, 2012). More than 90% of NAFLD patients have insulin resistance or feature metabolic syndrome (Ahmed *et al.,* 2015). Non-alcoholic steatohepatitis (NASH) is the most severe histological form of progress to cirrhosis with 20-30% of these patients experiencing a liver-related death (McCullough, 2006).

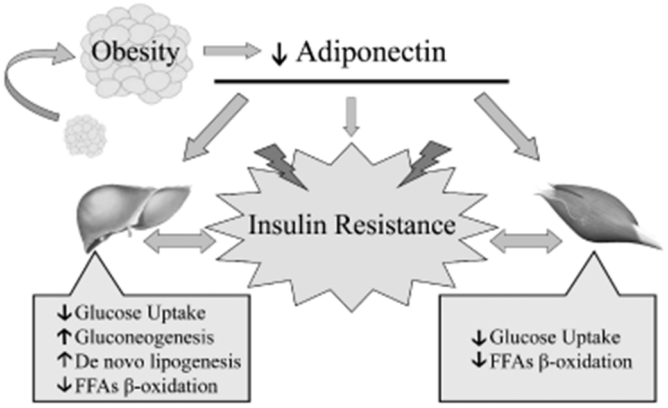

Figure C1-1. Schematic depicting the overall outcome of decreased circulating adiponectin levels.

Mechanisms contributing to NAFLD

NAFLD can result from insufficient adiponectin release from adipose tissue (Polyzos *et al.,* 2009). Adiponectin levels decrease as fat mass increases, thereby

resulting in subsequent signal transduction consequences within cells (Polyzos *et al.,* 2009). One of these consequences is insulin resistance (IR) (Polyzos *et al.,* 2009). With IR, glucose uptake is decreased and fatty acid oxidation are decreased while gluconeogenesis, *de novo* synthesis lipogenesis are increased creating a cycle of obesity, IR and the subsequent fatty liver (Polyzos *et al.,* 2009) (Figure C1-1). A dysregulation and an imbalance of adipokines/cytokines are abundantly found in NAFLD and have been shown to lead to IR and fatty liver (Watanabe *et al.,* 2008; Takei *et al.,* 2006; Jarrar *et al.,* 2008). Adiponectin is an adipose-specific adipokine that acts on amp-activated protein kinase (AMPK) within the liver protecting from insulin resistance (IR) and ultimately, fatty liver (Polyzos *et al.,* 2009) (Figure C1-2). Upon activation, AMPK down-regulates sterol regulatory element-binding proteins (Srebp), which are a group of membrane-bound transcription factors that regulate lipid synthesis (Kohjima *et al.,* 2008). Specifically, Srebp1c regulates the expression of genes encoding lipogenic enzymes such as acetyl-CoA carboxylase (Acc1) and fatty acid synthase (Fas) (Kohjima *et al.,* 2008). In NAFLD, as adiponectin is decreased,

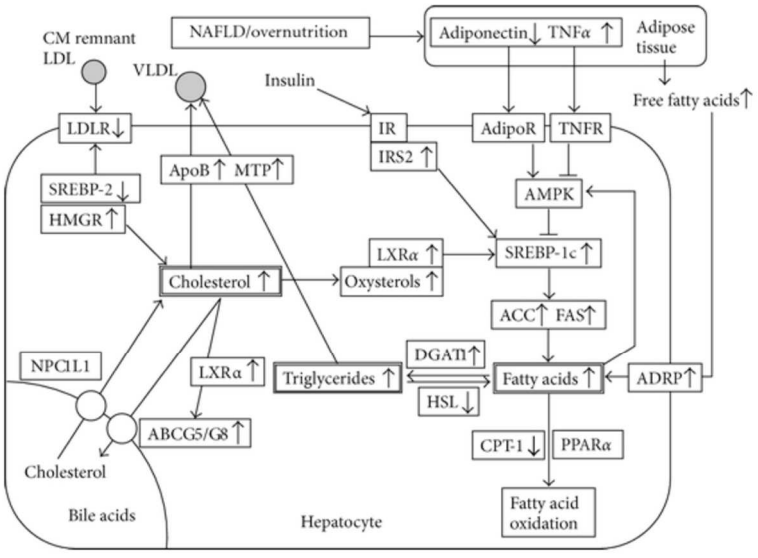

Figure C1-2: Schematic depicting molecular mechanisms contributing to NAFLD.

AMPK activation is also decreased resulting in Srebp1c activation and lipid synthesis via Acc1 and Fas within the liver eliciting a fatty liver (Kohjima *et al.,* 2008) (Figure C1-2).

Therapeutic strategies for NAFLD Treatment

The recommended therapeutic intervention to treat NAFLD is with diet and exercise, which have the ability to reverse hepatic lipid accumulation (Chalasani *et al.,* 2012). It has been shown that a calorically restricted (CR) diet with exercise decreases the hepatic steatosis and serum lipids in overweight patients with NAFLD (Larson-Meyer *et al.*, 2008). In earlier studies, it is suggested that CR with or without exercise decreases liver fat and liver enzymes with no difference between CR groups with the CR and exercise groups (Yoshimura *et al.* 2014; Straznicky *et al.,* 2012; Tamura *et al.,* 2005; Larson-Meyer *et al.*, 2008). In a randomized-controlled study trial of NAFLD patients, intervention with diet, after 12-months, 64% patients were in NAFLD remission which includes a reduction in body weight and total cholesterol levels (Wong *et al.*, 2013). NAFLD can be reversed with CR intervention.

Molecular Mechanisms by which CR decreases Liver Fat

The response to CR hinges upon a cellular metabolic shift in which AMPK is a central mediator. CR induces phosphorylation of AMP-activated protein kinase (AMPK) upon redox status (high AMP:ATP ratio) (Figure C1-3). AMPK is a regulator of hepatic metabolism in energy balance by promoting catabolic pathways and inhibiting ATP-consuming pathways and is a good target for the treatment of T2D (Viollet *et al.*, 2006). AMPK activation is implicated for the benefits of glucose and lipid metabolism with exercise, weight loss, and use of anti-diabetes drugs (Towler *et al.*, 2007).

AMPK inhibits hepatic glucose and lipid synthesis and induces lipid oxidation to produce energy (Nerstedt, 2010).

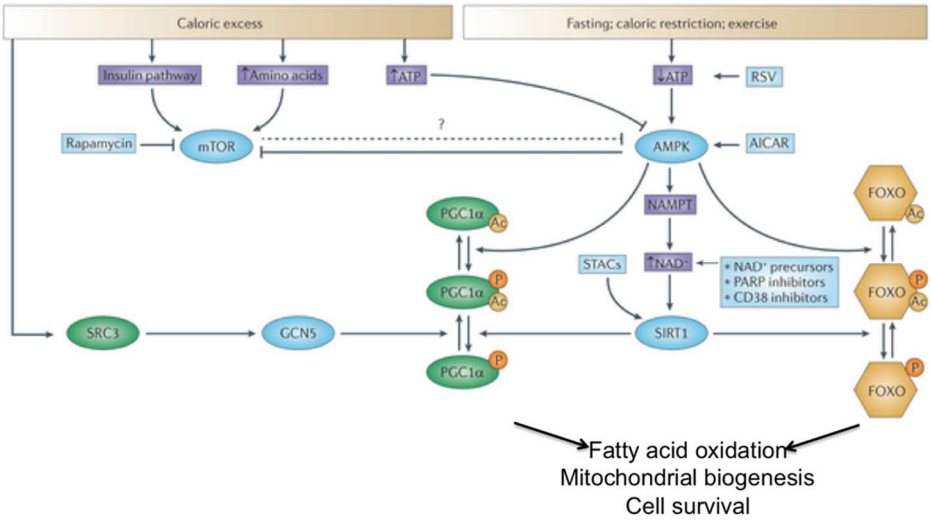

Figure C1-3: Schematic depicting molecular mechanisms in caloric restriction and excess.

In addition to caloric restriction, metformin has been examined as a pharmacologic therapeutic intervention for the management of NAFLD (Mazza *et al.,* 2012). Metformin was first introduced in the 1950's as a first-line T2D drug due to its ability to lower gluconeogenesis and ultimately blood glucose levels which increase glucose uptake within skeletal muscle as well as fatty acid oxidation within adipose tissue (Stumvoll *et al.* 1995) (Figure C1-4).

Metformin is used in the treatment of T2D and activates AMPK in hepatocytes, which in turn suppresses lipogenic genes, such as SREBP1c and Acetyl-CoA carboxylase (ACC) while increasing insulin sensitivity (Viollet *et al.* 2012; Zhou *et al.*, 2001).

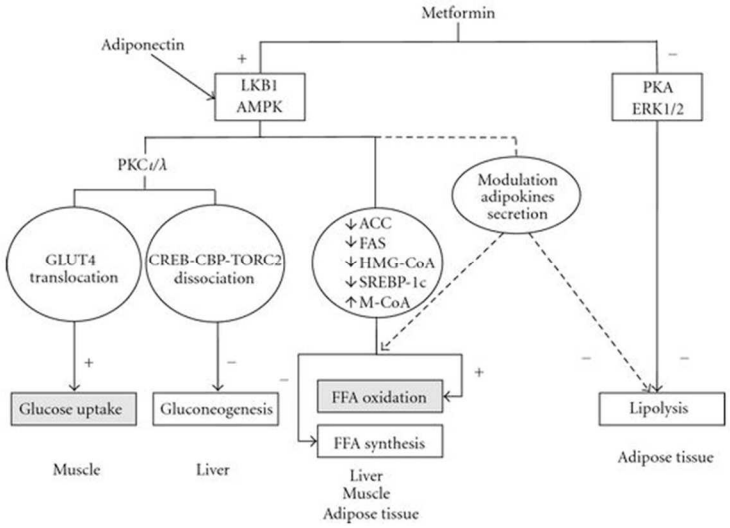

Figure C1-4. Schematic of the pathogenesis of metformin within liver and peripheral tissues.

Introduction to Perfluorinated Compounds:

Perfluorinated compounds (PFCs) are a group of synthetic anthropogenic organofluorines widely used in various industrial applications such as stain-resistant coatings for paper and fabric protection, leather, waxes, surfactants, emulsifiers, fire-fighting foams, textiles, chromium plating and more (Wan *et al.* 2012, Ziwen *et al.,* 2014). The main routes of human exposure are through ingestion, dermal exposure, inhalation and through drinking water (Saikat *et al.*, 2012). The main route of general population exposure to PFOS is via ingestion of fish and drinking water (Saikat *et al.,* 2012). There has been no evidence reported of the transformation of PFOS in soil, sediment or water (Saikat *et al.* 2012). Perfluorinated chemicals have been studied for a couple of decades and are known to induce fatty liver in rodents (EPA, 2009a; Hines, *et al.* 2009; Klaunig, *et al.* 2003; Tardiff*, et al.* 2009). Perfluorooctanesulfonic acid (PFOS) is a major 8-carbon PFC that is widely studied today. PFOS has been detected in the sera and tissues of animals, specifically fish, birds, and marine mammals, and humans worldwide (Giesy and Kannan, 2001; Calafat *et al.,* 2007; Lau *et al.,* 2007) and have been correlated

with adverse health effects in rodents and monkeys, including liver effects and decreased cholesterol (Lau *et al.,* 2007, Seacat *et al.,* 2002). PFOS has also been associated with energy metabolism disorders due to Ppar activation in rats (Bjork *et al.,* 2008). Environmental exposure of PFOA and PFOS to adult humans were associated with increased lipid levels (Eriksen *et al.,* 2013; Chateau-Degat *et al.,* 2010), however, in mice and monkeys, there is a negative correlation with cholesterol levels (Seacat *et al.,* 2003; Seacat *et al.,* 2002). Moreover, in adolescents and adults, increased serum PFOS concentration was associated with increased blood insulin, assessment of insulin resistance, and beta-cell function (Lin et al., 2009). Altered glucose and lipid metabolism has also been described in adult rats exposed to PFOS (Lv *et al.,* 2013).

Perfluorooctanoic acid (PFOA) is another PFC, but instead of the sulfonate moiety, it has a carboxylic acid. According to the EPA, PFOA has been investigated since the 1990s and is now a known liver toxicant (hepatomegaly), development toxicant, and carcinogen in rodents. It is detected in serum and tissues of wildlife and humans worldwide with an average half-life of 3.5 years in humans (EPA, 2009a; Hines, *et al.* 2009; Klaunig, *et al.* 2003; Tardiff*, et al.* 2009). Human studies of PFOA are limited, however, trace amounts of PFOA have been detected in adipose tissue (0.2ng/kg) (Calafat, *et al.,* 2007). It is known that PFOA accumulates mostly in liver and kidney and is detectable in serum (Hundley *et al.,* 2006; Olsen *et al.*, 2005). PFOA exposure has resulted in weight loss with liver injury and lipid metabolism disorders in mice (Seacat *et al.,* 2002; Jensen *et al.,* 2008).

Levels of PFOA and PFOS were detected in non-occupationally exposed general population humans of liver, kidney, adipose tissue, brain, basal ganglia, hypophysis, thyroid, gonads, pancreas, lung, skeletal muscle and blood and found PFOA ranging from 0.3 to 3.8ng/g and PFOS ranging from 1.0 to 13.6ng/g with the highest concentrations within the liver (Maestri *et al.,* 2006). Low doses of PFOA and PFOS have still been suggested to increase hepatomegaly (Wan *et al.,* 2012; Yan *et al.,* 2014), which may be due to the induction of peroxisome proliferation through the agonistic nature of PFOA and PFOS on Ppar's (Pyper *et al.,* 2010; Lau *et al.,* 2007; Takacs and Abbott, 2007). However, studies with PFOA administration in Ppar-α-null mice still observed increased liver weight and changes in gene expression associated with fatty acid metabolism and fat accumulation suggesting Ppar-independent mechanisms (Minata *et al.,* 2010; Wolf *et al.,* 2008; Rosen *et al.,* 2008a and b).

The purpose of these following dissertation studies was to evaluate PFOS and PFOA on metabolic tissues of mice treated with these compounds. The overall goal of Manuscript I was to determine whether PFOS exposure interferes with recommended therapies to decrease hepatic lipid content that are used to treat NAFLD. It was hypothesized that PFOS administration could interfere with the benefits of CR and metformin potentially through targeting AMPK phosphorylation. Overall, we present novel findings illustrating that PFOS administration concurrent with a modest reduction in caloric intake thwarted CR-induced decline in hepatic lipids *in vivo* and improvement in glucose tolerance and interfered with metformin-induced glucose lowering effects *in vitro*. The goal of Manuscript II was to determine whether PFOA treatment affects fatty acid oxidation, lipid synthesis, and antioxidant response gene

expression in adipose tissue. Results indicate no significant findings of PFOA affecting any gene expression within adipose tissue of mice after a 7-day treatment with 1.0 or 3.0 mg/kg/day.

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Chapter 2

MANUSCRIPT I

Perfluorooctanesulfonic acid (PFOS) thwarts the beneficial effects of caloric restriction and metformin

Deanna M. Salter*, Wei Wei*, Pragati P. Nahar* and Angela L. Slitt*,[1]

*Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, RI, 02881, USA

Running title: PFOS interferes with caloric restriction

Manuscript in Preparation

[1]**Corresponding Author:**
Angela L. Slitt, Ph.D.
Department of Biomedical and Pharmaceutical Sciences
University of Rhode Island
7 Greenhouse Road, Kingston, RI 02881
Phone: (401) 874-5020
Fax: (401) 874-5048
E-mail: aslitt@uri.edu

ABSTRACT

A combination of caloric restriction (CR), dietary modification, and exercise is the recommended therapy to reverse obesity and nonalcoholic fatty liver disease. The ability to mount an effective response to caloric restriction required to effectively shift hepatic metabolism to fatty acid oxidation depends upon induction of pathways, such as AMP- activated kinase (AMPK). PFOS, a fluoro-surfactant previously used as a stain repellent and anti-stick material increases hepatic lipids in mice following relatively low dose exposures. We hypothesized that PFOS administration will interfere with CR-induced reduction of hepatic lipids and improve glucose tolerance. Adult male C57BL/6 mice were fed *ad libitum* or 25% reduced calorie diet concomitant with either vehicle (water) or 100 μg PFOS/kg/day for 6 weeks. PFOS did not significantly alter CR-induced weight loss or WAT mass or liver weight over 6 weeks. However, PFOS increased hepatic triglyceride accumulation, and hepatic triglycerides were higher in PFOS-treated mice that subjected to calorie restriction, which was associated with decreased phosphorylated AMPK protein levels in livers of CR mice. PFOS interfered with CR-induced improvement of glucose tolerance with decreased IRS-1 mRNA expression in liver. PFOS induced glucose production in hepatocytes and HepG2 cells, which was partially antagonized by the AMPK activating compound, AiCAR. Moreover, metformin suppression of gluconeogenesis was not as robust in HepG2 cells with PFOS co-treatment. Overall, PFOS administration had disruptive effects for hepatic lipids and glucose homeostasis, and interfered with beneficial glucose lowering effect of metformin.

Key words: Liver, skeletal muscle, PFOS, perfluorinated compounds, caloric restriction, AMP-activated protein kinase, NAFLD, Metformin

INTRODUCTION

Obesity and T2D are concurrent, and predispose individuals to development of fatty liver disease, referred to as non-alcoholic fatty liver disease (NAFLD) (Li *et al.*, 2002). NAFLD is a complex multifactorial disease in the U.S. with 10% of children, 30% of adults, 50% of obese, 76% of diabetics and 100% of morbidly obese diabetics affected (Eguchi *et al.*, 2013; Naik *et al.*, 2013; Reddy and Roa, 2006). It is characterized by the accumulation of lipids in the liver, lipotoxicity and insulin resistance and thus frequently found in people with central obesity or diabetes (Naik *et al.*, 2013; Smith and Adams, 2011). NAFLD as a disease is considered to be a spectrum – ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). Steatosis is the accumulation of triglycerides within the hepatic parenchymal cell cytoplasm, whereas non-alcoholic steatohepatitis (NASH) consists of classical steatosis accompanied by inflammation (Naik *et al.*, 2013; Reddy and Roa, 2006). Hepatic lipid accumulation has been shown to lead to decreased insulin sensitivity, ultimately leading to insulin resistance, commonly observed in diabetes (Montell *et al.*, 2001). A hallmark characteristic of hepatic steatosis is the disequilibrium between *de novo* lipogenesis, free fatty acid uptake, oxidation, esterification and secretion of lipids from the liver (Naik *et al.*, 2013). Hepatic lipid accumulation can be attributed to dampened lipid utilization via altered mitochondrial oxidation and hepatic lipid export as well as excess adiposity and decreased insulin sensitivity (Smith and Adams, 2011). Multiple factors are suggested to contribute to NAFLD and environmental

exposure has been hypothesized as a potential risk factor for predisposition to NAFLD (Cave *et al.,* 2007).

The American Association for the Study of Liver Disease NAFLD treatment guidelines recommends dietary modification and/or weight loss as the current NAFLD therapeutic strategy (Larter *et al.*, 2013). The recommended therapeutic intervention to treat NAFLD is with diet and exercise, which have the ability to reverse hepatic lipid accumulation (Chalasani *et al.,* 2012). It has been shown that a CR diet with exercise decreases the hepatic steatosis and serum lipids in overweight patients with NAFLD (Larson-Meyer *et al.*, 2008). A diet with 30% caloric restriction without malnutrition delays the onset of multiple diseases such as diabetes, cancer and cardiovascular disease in Rhesus monkeys (Colman *et al.* 2009). A calorie restricted (CR) diet in humans on average for 6 years improved metabolism and insulin levels (Fontana *et al.*, 2004). In human studies, diet and exercise were effective in the reduction of NAFLD (Oh *et al.*, 2014; Schwenger and Allard, 2014). In addition, caloric restriction mimetics have been examined as potential treatments for NAFLD. For example, Metformin, a commonly used anti-diabetes drug with some CR mimetic properties has been investigated as a potential therapy to treat NAFLD (Maslak *et al.,* 2014), with some positive results (Maslak *et al.,* 2014).

CR increases the cellular AMP:ATP ratio, which induces AMP-activated

protein kinase (AMPK) activation (Fulco and Sartorelli, 2008). AMPK is a heterotrimeric serine/threonine protein kinase that regulates energy homeostasis in metabolic tissues upon high AMP:ATP ratios with phosphorylation at the Thr 172 residue of the alpha subunit (Nerstedt, 2010). AMPK activation is implicated for the benefits of glucose and lipid metabolism with exercise, weight loss, and use of anti-diabetes drugs (Towler *et al.*, 2007). AMPK inhibits hepatic glucose and lipid synthesis and induces lipid oxidation to produce energy (Nerstedt, 2010). AMPK phosphorylates sterol regulatory element binding protein-1c (SREBP-1c) at Ser-327 inhibiting its activity, diminishing SREBP1c-mediated lipogenesis reducing hepatic steatosis (Li *et al.*, 2013). CR increases phosphorylation of AMPK, which then activates Sirt1 deacetylase under certain redox status (Fulco and Sartorelli, 2008). In steatotic livers, there is a decrease in AMPK phosphorylation in rodents (Ha *et al.*, 2011). Under CR, four overweight males displayed increased phosphorylated AMPK and SIRT1 in peripheral blood mononuclear cells (Kitada *et al.*, 2013). AMPK is a regulator of hepatic metabolism in energy balance by promoting catabolic pathways and inhibiting ATP-consuming pathways and is a good target for the treatment of T2D (Viollet *et al.*, 2006). Metformin is used in the treatment of T2D and activates AMPK in hepatocytes, which in turn suppresses lipogenic genes, such as SREBP1c and Acetyl-CoA carboxylase (ACC) (Viollet *et al.* 2012; Zhou *et al.*, 2001). 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR), an AMPK activator, also reversed alcoholic fatty liver in rats (Tomita *et al.*, 2005). Both Metformin and

AICAR act on AMPK to provoke similar effects to increase ATP-producing pathways and elicit beneficial results (Zhou *et al.*, 2001; Tomita *et al.*, 2005). Perfluorinated chemicals (PFCs) have been studied for a couple of decades and have multiple detrimental effects in rodents (Lau *et al.,* 2007). Specifically, PFOS, According to the Environmental Protection Agency (EPA), PFOS is considered to be a hepatotoxicant and obesogen and possible carcinogen in humans. There are limited data regarding PFOS effects in human related to NAFLD or liver effects. The C8 Health Project, which has studied a cohort of about 47,000 human subjects, and reported a positive correlation between serum PFOS and ALT concentration, suggesting liver effects (Gallo, 2012). PFOS administration is described to increase liver weight, expression of genes in fatty acid oxidation in rodents (Wan *et al.*, 2012). Moreover, PFOS-induced steatosis has been shown to be dose- and time- dependent, as observed with increased expression of fatty acid transport genes, and decreased expression mitochondrial β-oxidation genes (Wan *et al.*, 2012). Liver weight increases are, in part, attributed to an increase in hepatic triglycerides (Bijland *et al.*, 2011). The reversibility of PFOS-induced hepatic lipid accumulation has not been described.

The overall goal of this project was to determine whether PFOS exposure interferes with recommended therapies to decrease hepatic lipid content that are used to treat NAFLD. It was hypothesized that PFOS administration could interfere with the benefits of CR and metformin potentially through targeting

AMPK phosphorylation. Overall, we present novel findings illustrating that PFOS administration concurrent with a modest reduction in caloric intake thwarted CR-induced decline in hepatic lipids and improvement in glucose tolerance and interfered with metformin-induced glucose lowering effects *in vitro*.

MATERIALS AND METHODS

Animals. 10-week-old male C57BL/6 mice weighing approximately 30 grams were purchased from Charles River Laboratories (Wilmington, MA). The mice were housed under a controlled temperature (70-73F) with relative humidity (30-70%), lighting (12 h, light-dark cycles) environment and acclimated for 5 weeks on the standard rodent chow to allow for additional weight gain. At 15 weeks of age, the mice were then fed a purified rodent chow (AIN-93G Growth Purified Diet, TestDiet, St. Louis, MO) and monitored for an additional 5 weeks for food consumption to calculate a 25% calorie restriction. At 21 weeks of age, mice were fed either *ad libitum* (AL) or placed on a 25% caloric restriction (CR). The mice were then divided into two subsequent groups (n=8) that were administered tap water as vehicle (Veh) via oral gavage (5 ml/kg) or Heptadecaperfluorooctanesulfonic acid (PFOS) potassium salt (100 μg/kg, 5 ml/kg) purchased from Sigma Aldrich (catalog#77282, Lot#BCBH2834V, St. Louis, MO) for 6 weeks. Body weight and food intake were monitored daily and recorded. Serum, liver, white adipose tissue, and skeletal muscle from non-fasted mice were collected, snap frozen with liquid nitrogen, and stored at -70°C until analysis.

Glucose tolerance test and insulin tolerance test. Glucose tolerance (GTT) and insulin tolerance tests (ITT) were performed as previously described (More *et al.,* 2013; Xu *et al.,* 2012). The GTT and ITT were performed after 3 and 4 weeks of CR and PFOS treatment, respectively. For the GTT, mice

were fasted for 12 hours and glucose (1 g/kg, i.p.) was administered. For ITT, mice were fasted for 12 hours and insulin (1 unit/kg, i.p.) was administered. Blood glucose was determined by measuring tail blood concentrations at 0, 15, 30, 60, and 120 min after glucose or insulin administration using a Bayer Contour glucometer.

Measurement of cholesterol, triglyceride, free fatty acid, and glucose concentration. Cholesterol, triglyceride, and, free fatty acid quantifications were performed using colorimetric assay kits from Pointe Scientific Inc. (Canton, MI) according to the manufacturer's protocol. Tissues (50 mg) were homogenized with PBS and extracted with chloroform-methanol (2:1; vol/vol). The residue was re-suspended in 1% Triton X-100 in 100% ethanol. Lipid content was normalized with tissue weight. Serum glucose concentration was measured with a glucose assay kit (Eton Bioscience Inc., San Diego, CA) based on manufacturer's protocol.

RNA isolation and quantitative real-time PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Camarillo, CA) according to the manufacturer's instructions. 1 μg of total RNA was converted to cDNA, and mRNA levels were quantified by quantitative real-time PCR using a Roche LightCycler 480 System (Roche Applied Science, Mannheim, Germany). SYBR green reagent was used, and relative target gene expression was normalized to GAPDH mRNA.

Determination of relative protein expression in liver and skeletal muscle by western blot. Tissues (~50 mg) were homogenized in 1 ml of RIPA buffer containing protease and phosphatase inhibitor. Protein lysates were electrophoretically resolved using polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were resolved by SDS-PAGE (10% resolving and 4% stacking gel) and then transblotted onto low fluorescence polyvinylidene fluoride membrane (PVDF) (Millipore, Billerica, MA) in transfer buffer at 30V overnight. Antibody blocking conditions, dilutions, incubation conditions, and secondary antibodies are described in a supplemental table (Supplemental Table 1). PVDF membranes were incubated in blocking solution for 1 hour. Next, blots were incubated with primary antibody specific to the protein of interest diluted in blocking buffer at either 4°C overnight or room temperature for 3 hours. Primary antibody was removed and the membrane was washed with washed in tris-buffered saline with 0.2% Tween 20 (TBS/T) three times for 5 min at room temperature. Next, blots were incubated with secondary antibody specific diluted in blocking buffer for 1 hour. The membrane was either visualized if infrared detection was used (LiCOR Odessey, Lincoln,NE) or incubated in ECL+ chemiluminescent substrate for 5 min (GE Healthcare Life Sciences, Pittsburgh, PA) and visualized using light-sensitive film. The relative abundance of protein in each sample was quantified and normalized to β-actin.

Primary hepatocyte and HepG2 treatment and assay for glucose production. *Primary mouse hepatocyte isolation*. Primary mouse hepatocytes were isolated from livers of adult C57BL/6 mice using a two-step collagenase perfusion; $1x10^6$ cells/well in 2 mL completed medium (William's Medium E supplied with 10% FBS) were seeded on collagen-coated 6-well plates. After cell attachment (~4 h), they were cultured in serum-free William's E Media containing 1% insulin-transferrin-selenium (ITS) and dexamethasone supplement (Invitrogen, CA). Media was then replaced with custom William's Media E lacking glucose and supplemented with pyruvate. Media was collected at time zero to measure glucose output. Glucagon (100nM), PFOS (2.5 μM) or a combination of both, were added to the hepatocytes and media was collected at 6 hours to measure glucose output using a glucose assay (Eton Bioscience Inc., San Diego, CA). HepG2 cells were treated with PFOS (25, 50, and 100 μM), and metformin (1mM) alone or in combination. Media was collected after 6-10 hours after treatment and media glucose concentration was assayed using the glucose assay described above.

Adiponectin assay. The Mouse Adiponectin/Acrp30 Quantikine ELISA Kit was purchased from R&D Systems, Minneapolis, MN. The protocol followed was according to the product insert. Mouse sera was diluted 1:2000 and media from cultured adipocytes was diluted 1:2000 before running in the assay.

Oil-Red-O Staining. Cells were fixed with 10% buffered formalin for 30 min and then stained with Oil red O (ORO) working solution. A fresh working solution was prepared by combining 3 parts of stock solution (0.5% ORO in isopropanol) with 2 parts of deionized water and then filtered after 15 minutes. Cells were incubated in working solution for 15 min at room temperature and then ORO solution was removed. Wells were quickly rinsed 2 times with fresh 60% isopropanol, washed with water, and mounted in glycerin jelly. Quantification includes the addition of 1mL isopropanol to each well then 140uL of those wells into a 96-well plate for spectrophotometer reading.

Statistical Analysis.

Power analysis using the sample size calculator http://www.jerrydallal.com/LHSP/SIZECALC.HTM was performed with the following assumption that the difference in mean between the test and control group is 50% of the mean and the standard deviation is 25% of the mean values. Based on this analysis, the sample size used was an n = 8. Depending on the analysis, an unpaired Student's T-test was used or an ANOVA followed by a Duncan's Multiple Range *Post Hoc* Test. Significance was considered to be $p<0.05$.

Results

Effect of CR and PFOS on body weight, liver weight, and serum chemistry. 21-week old male C57BL/6 mice were fed *ad libitum* or 25% calorically restricted for 5 weeks. In each group, mice were administered vehicle (VEH) or 100 μg/kg PFOS daily. In Figures 1a and b, CR reduced the percent body weight from day 0 and the AUC for body weight over time. Body weight and percent weight loss was similar between VEH and PFOS treated mice over the course of the study. CR decreased liver weight by 37.4% and WAT tissue by 48.2% (Figures 1C & D). Liver and WAT weight was similar between VEH and PFOS treated groups. Overall, PFOS administration did not significantly alter body or tissue weight, as well as response to CR-induced weight loss.

Serum clinical markers associated with response to weight loss and CR were evaluated (Table 1). CR increased adiponectin levels by 24.2% compared to AL fed mice. In AL fed mice, PFOS also increased adiponectin by 23.4%. In CR fed mice, PFOS did not significantly affect serum adiponectin levels. CR decreased serum glucose by 29.6% compared to AL fed mice. Interestingly, in AL fed mice, PFOS increased serum glucose levels by 65.3% compared to AL control. In CR fed mice, PFOS increased serum glucose levels by 67.3% compared to CR mice administered vehicle.

PFOS interfered with CR-induced improvement of glucose utilization.

CR improves glucose tolerance and increases insulin sensitivity (Colman *et al*., 2009; Fontana, 2009). The effect of PFOS on this CR-induced benefit was evaluated. CR decreased glucose levels over time (Figures 2a and b). Overall, CR decreased glucose load by 57.9% compared to AL fed mice (Figure 2c). In AL fed mice, PFOS administration did not significantly affect response to glucose challenge, although the glucose load tended to increase, but did not reach statistical significance (p=0.8938) (Figures 2a-c). Interestingly, PFOS administration did affect the response to glucose challenge in mice that were placed on CR, with glucose clearance being decreased in CR mice administered PFOS (Figure 2a and b). Compared to CR mice administered VEH, glucose load was 74.3% higher in CR mice administered PFOS (Figure 2c). In CR and AL fed mice, PFOS significantly increased the glucose load after 2.5 hours after insulin challenge compared to controls (Figure 2d), suggesting that PFOS interferes with insulin suppression of hepatic glucose production. Glucose homeostasis hepatic gene expression was analyzed by qPCR. CR did not markedly decrease Irs1 gene expression levels, however, with PFOS, CR fed mice significantly decreased Irs1 gene expression (Figure 2e). CR fed mice had decreased hepatic gene expression of Pepck and G6pase compared to AL controls (Figure 2e). In both CR and AL fed mice, PFOS significantly decreased Glut2 gene expression (Figure 2e). Protein quantification of phosphorylated-akt was measured within the lysates of

skeletal muscle of these mice. PFOS had no affect on p-akt in either CR or AL fed mice (Figure 2f).

PFOS significantly increased lipid content in the livers of mice.

It has been previously reported that PFOS increased liver weight (Seacat *et al.*, 2002; Seacat *et al.*, 2007) and this may be attributable to an accumulation of lipids within the liver (Polyzos *et al.*, 2009). We evaluated the liver histology with hematoxylin and eosin (H&E) staining. CR There is the presence of vacuoles in the CR-PFOS treatment group that are not seen in any other group (Figure 3a). PFOS significantly decreased hepatic free fatty acids (FFA) by 38.7% in AL fed mice and 39.2% in CR fed mice (Figure 3b). These data are further supported by the significant increase in triglycerides in PFOS-treated mice. As expected, CR fed mice had a significant 31.9% decrease in hepatic triglycerides compared to AL control (Figure 3c). Interestingly, PFOS significantly increased hepatic triglycerides in both AL and CR fed mice by 94.9% compared to AL-Veh and 37.6% compared to CR-Veh, respectively (Figure 3c). CR fed mice significantly increased hepatic gene expression of Cyp4a14 compared to AL fed control (Figure 3d). PFOS significantly increased gene expression in the liver of Cyp4a14 in AL and CR fed mice compared to controls (Figure 3d). PFOS significantly decreased Ppar-α hepatic gene expression in both AL and CR fed mice compared to controls (Figure 3e). Downstream targets of Ppar-α were increased upon CR with

significant induction of Gpat hepatic gene expression with PFOS exposed CR fed mice (Figure 3e).

PFOS alters glucose homeostasis protein expression of mice.

To better understand whether PFOS was affecting response to CR at the level of protein expression, liver lysates were evaluated for changes in protein expression. Western blot analysis of proteins involved in glucose and lipid homeostasis was measured (Figure 4a and b). CR fed mice with PFOS administration protein levels were compared to CR fed mice with vehicle (Figure 4a). Phosphorylated-akt is responsible for the uptake of glucose into cells and is significantly decreased 38.4% upon PFOS administration in mice that underwent CR compared to CR control (Figure 4c). Conversely, Phosphoenolpyruvate carboxykinase (Pepck) is the main regulator of gluconeogenesis and is significantly increased, both supporting the glucose data in Figure 2 (Figure 4c). Protein levels of P-ampk decreased significantly by 34.2% upon PFOS administration with CR compared to control (Figure 4c). Protein levels in all groups were compared (Figure 4b). Cluster of differentiation (CD36), also known as fatty acid translocase (FAT), and fatty acid synthase (Fas) both increased significantly upon PFOS exposure with CR fed mice by over 2-fold and 2.5-fold, respectively, compared to CR control (Figure 4d). Astoundingly, P-ampk significantly decreased over 7-fold in mice that underwent CR with PFOS administration compared to CR control (Figure 4d).

PFOS significantly increased glucose within the media of Primary Hepatocytes and HepG2 cells.

Based on our GTT observations, and changes in P-AMPK in liver, the effect of PFOS on glucose production in hepatocytes was measured. Primary hepatocytes were isolated from C56BL/6 mice and were treated with glucagon, PFOS (2.5uM) and a combination of both for 24 hours. PFOS increased glucose levels in media of primary hepatocytes eliciting a glucagon-like effect. Expectedly, glucagon increased glucose within the media of HepG2 cells 13-fold compared to vehicle (Figure 5a). PFOS also increased the glucose within the media by 15-fold and taken together, PFOS and glucagon treatment increased the glucose within the media by 19.6-fold compared to vehicle (Figure 5a). HepG2 cells were treated for 10 hours with Metformin (1mM), PFOS (25uM or 100uM) and a combination of both. Metformin significantly decreased glucose production in the media of HepG2 cells by 67.2% compared to DMSO control (Figure 5b). PFOS (100uM) significantly increased glucose within the media by 3.2-fold compared to DMSO control (Figure 5b). When challenged with metformin, PFOS still induced glucose output into the media of HepG2 cells by 7-fold compared to metformin control (Figure 5b), illustrating that PFOS dampens the Metformin-induced lowering of glucose within the media. In a dose-response study with HepG2 cells, 5-aminoimidazole, 4-carboxamide ribonucleotide (AICAR) was used to stimulate phosphorylation of AMPK and challenged with different

concentrations of PFOS (Figure 5c). PFOS increased glucose production at 25uM 6-fold compared to DMSO control (Figure 5c). PFOS (25uM) with AICAR increased glucose within the media by 3.5-fold compared to AICAR control (Figure 5c). P-ampk protein levels were analyzed by western blot analysis from the lysates of data in Figure 5b. Metformin increased p-ampk while PFOS (25uM) decreased p-ampk protein levels. When treated with metformin and PFOS (25uM), p-ampk levels are greatly decreased compared to metformin control (Figure 5d).

PFOS increases glucose within the media of 3T3L1 cells and human adipocytes.

Mature adipocytes were treated with Metformin (1mM) and PFOS (50uM) or in combination for 12 hours. PFOS significantly increased glucose within the media of these cells compared to the DMSO control (Figure 6a). Although there is an increasing trend of PFOS with metformin, this is not statistical (Figure 6a). Adiponectin levels were also measured, however, no changes were observed among groups (Figure 6b). Human adipocytes were treated with Metformin (1mM), PFOS (50uM) or in combination for 24 hours (Figure 6c). PFOS showed an increasing trend of glucose within the media, however this is not statistical. PFOS in combination with Metformin significantly decreased the glucose in the media of these cells compared to DMSO control (Figure 6c). Oil-Red-O staining of human adipocytes shows more lipids with PFOS treatment compared to DMSO control (Figure 6d).

Discussion

NAFLD is a rising worldwide and is associated with obesity and diabetes (Masarone *et al.*, 2014). Diabetes and NAFLD predict the development of one another and create a cycle of progression from NAFLD to diabetes and vice versa (Williams *et al.*, 2013). NAFLD is characterized by >5% of the liver made up of fat, lipotoxicity and insulin resistance and thus frequently found in people with central obesity or diabetes (Naik *et al.*, 2013; Smith and Adams, 2011). Fatty liver is a status of insulin-resistance and responds to treatments originally developed for other insulin-resistant states such as Metformin (Ford *et al.,* 2015). Obesity is considered to be a predominant risk factor for NAFLD, however other etiologies for NAFLD have been hypothesized, such as environmental factors (Cave *et al.,* 2007). The purpose of this study was to evaluate whether, PFOS, an environmental chemical associated with adverse liver effects would affect the recommended therapeutic strategy to treat NAFLD. Our findings herein illustrate that PFOS dampened the response to CR and interfered with metformin-induced glucose consumption.

The most beneficial treatment of NAFLD is a low-fat diet and exercise (Chalasani *et al.,* 2012). In earlier studies, it is suggested that CR with or without exercise decreases liver fat and liver enzymes with no difference between CR groups with the CR and exercise groups (Yoshimura *et al.* 2014; Straznicky *et al.,* 2012; Tamura *et al.,* 2005; Larson-Meyer *et al.*, 2008). It has been reported that a 15, 30 and 40% kCal reduction in mice has beneficial implications on body weight and hepatic lipids (Zidong and Klaassen, 2013).

In our study, mice were placed on a 25% kCal reduction or fed *ad libitum* for 5 weeks dosed with vehicle or challenged with 0.1mg/kg/day PFOS. Expectedly, our results are consistent with other studies showing significant decreases in body, liver and WAT weights with a ~25%kCal reduction (Weindruch *et al.,* 1986; Zidong and Klaassen, 2013) which is on the lower side of a kCal reduction (ranges from ~25-60%) (Koubova and Guarente, 2003). Our glucose tolerance test (GTT) provides evidence for CR increasing glucose tolerance and lowering the total glucose load within mice as well as significantly decreasing serum and hepatic lipids CR and these results are consistent with other studies (Zidong and Klaassen, 2013). AMPK inhibits hepatic glucose and lipid synthesis and induces lipid oxidation to produce energy (Nerstedt, 2010). AMPK is a regulator of hepatic metabolism in energy balance by promoting catabolic pathways and inhibiting ATP-consuming pathways and is a good target for the treatment of Type II diabetes (Viollet *et al.*, 2006). AMPK activation is implicated for the benefits of glucose and lipid metabolism with exercise, weight loss, and use of anti-diabetes drugs (Towler *et al.*, 2007). Expectedly, in our study, CR induced AMPK activation in mice compared to AL control. AMPK is a heterotrimeric serine/threonine protein kinase that regulates energy homeostasis in metabolic tissues upon high AMP:ATP ratios (Nerstedt, 2010).

It is known that PFCs elicit fatty liver, but fewer studies address effects of PFOS on hepatic lipid accumulation. To the best of our knowledge, this is the first study that has investigated the effects of PFOS on the role of Metformin

and AMPK in a calorically restricted diet. Here, we show that a daily dose of PFOS for over five weeks dampens the beneficial lipid loss of CR as well as decreasing the ability of Metformin to lower glucose production via AMPK. PFOS exposure has been shown to increase serum glucose and insulin levels, although not statistically (Wan *et al.,* 2014). Wan *et al.* also show a pre-diabetic phenotype within mice exposed to PFOS (Want *et al.*, 2014). We show the same trend where PFOS exposure leads to increasing glucose within the serum of in vivo and in vitro models. IRS-1 gene expression was significantly decreased upon PFOS exposure in mice undergoing CR compared to our *ad libitum* mouse model. Glut-2 gene expression was significantly decreased in both CR and AL mouse models upon PFOS exposure, which may be due to glucose-intolerance as seen with the GTT. In this study, we used the C57BL/6 mouse model on a purified diet that underwent CR and daily administration of PFOS to assess the effects of PFOS on a CR diet via liver physiology. We recognize that this model is in lean mice and is not a true clinical representation of individuals undergoing CR and an obese mouse model would be the better representation of studying PFOS effects on NAFLD compared to CR mice with PFOS exposure to truly assess PFOS liver physiology. PFOS has been shown to increase body and liver weight but has also elicited effects in models in which did not show an increase in body or liver weight (Ngo *et al.*, 2014; Nelson *et al.*, 2010). A study showed a decrease in body weight but an increase in liver weight with PFOS exposure (Seacat *et al.*, 2003). Another study showed increased body weight

at a lower concentration of 1 mg/kg and increased body weight at higher concentrations of 5 and 10mg/kg over 21 days with all concentrations increasing the liver weight attributed to the appearance of cellular vacuolations (Wan *et al.*, 2012; Kim *et al.,* 2011). In our model, a sub-chronic dose of PFOS did not affect the body or liver weights of mice with or without CR. PFOS has been shown to have a positive correlation with serum ALT levels (Gallo, 2012) and increased liver weights (Seacat *et al.*, 2014), which may be attributable to an accumulation of lipids. Associations have been made here PFOS has increased the number and size of cytoplasmic vacuoles as well as and increase in lipid content, concluding the vacuoles contain lipids (Wan *et al.*, 2012). In our study, we show PFOS increasing hepatic triglycerides, interfering with the CR-induced lipid loss. H&E staining shows PFOS with CR induces the presence of vacuoles compared to any other group and it is reasonable to assume that these vacuoles contain lipids. PFOS induces PPAR-α, which is suggested to account for toxicity associated with PFOS (Takacs and Abbott, 2007), however, there are PPAR-α independent toxicities as well (Rosen *et al.,* 2010). We show induction of CYP4a14, a downstream target of PPAR-α, upon PFOS exposure.

We used Metformin as a treatment in *in vitro* models because it is used in the treatment of Type II diabetes and has been shown to activate AMPK (Zhou *et al.*, 2001). We show that PFOS dampens the beneficial effects of Metformin on lowering glucose production via AMPK activation. Our results indicate that

PFOS can potentially interfere with AMPK phosphorylation in liver and cells, which is relatively new to the field of environmental health. For example, we observed that glucose production was increased in HepG2 cells and hepatocytes treated with PFOS. AMPK suppression is known to induce gluconeogenesis via LKB1 down-regulation (Shaw *et al.,* 2005). In a study, genetic silencing of LKB1 resulted in nearly a complete loss of AMPK activity (Shaw *et al.,* 2005), suggesting LKB1 is necessary for AMPK activation. PFOS is suggested to be transported into the liver via Oatp2 or Oatp1d1 (Popovic *et al.,* 2014; Yu *et al.,* 2011). The primary transporters required for hepatic uptake of Metformin are OCT1 and OCT3 (Zheng *et al.,* 2014). PFOS and Metformin do not utilize the same transporters, suggesting that PFOS may inhibit beneficial effects of Metformin downstream.

Glucose in the media of primary hepatocytes was statistically increased upon PFOS and glucagon exposure. Metformin significantly decreased glucose in the media while PFOS (100 μM) significantly increased media glucose in HepG2 cells after 10 hours of exposure. A combination of Metformin and PFOS 100 μM was increased compared to Metformin alone. Both Metformin and AICAR act on AMPK to elicit similar effects to decrease ATP-consuming pathways and increase ATP-producing pathways (Zhou *et al.*, 2001; Tomita *et al.*, 2005). Both PFOS (25 μM) and AICAR in combination with PFOS (25 μM) significantly increase glucose in media compared to DMSO control in HepG2 cells. AICAR with PFOS (25 μM) significantly increases glucose in media compared to AICAR alone. P-AMPK protein levels were measured in HepG2

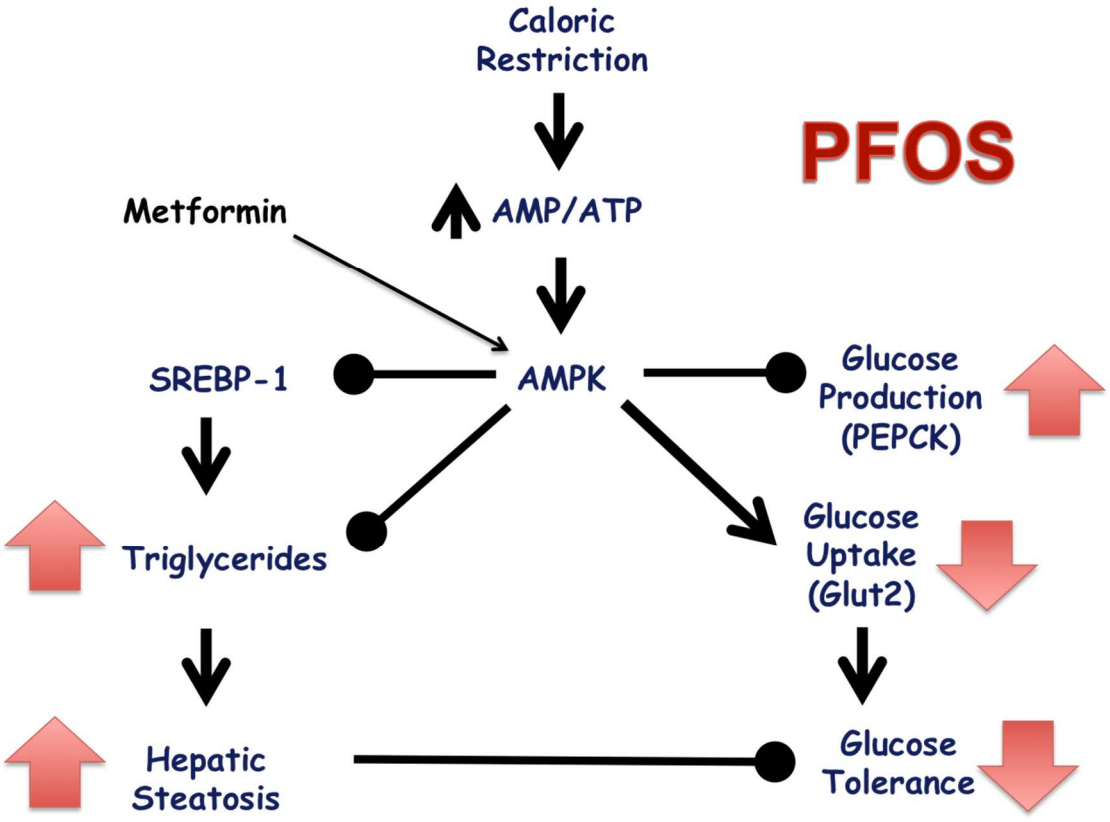
cells treated with PFOS (25 and 50 μM) and Metformin (1mM) where PFOS decreases P-AMPK protein levels when co-treated with Metformin, diminishing beneficial effects of Metformin.

In our study, we present novel findings illustrating that PFOS administration concurrent with a modest reduction in caloric intake thwarted CR-induced decline in hepatic lipids and improvement in glucose tolerance and interfered with metformin-induced glucose lowering effects *in vitro*. To the best of our knowledge, this is the first study that investigates PFOS effects on metformin and AMPK in a calorically restricted diet. Our results indicate novel findings of lowered AMPK levels upon PFOS administration as well as PFOS interference with a major pharmacological therapeutic for diabetic intervention.

Conclusion

Perfluorooctanesulfonic acid, a fluoro-surfactant previously used as a stain repellent and anti-stick material increased hepatic lipids in mice following relatively low dose exposures. An increase in hepatic lipids leads to non-alcoholic fatty liver disease. A combination of caloric restriction, dietary modification, and exercise is the recommended therapy to reverse obesity and nonalcoholic fatty liver disease. The ability to mount an effective response to caloric restriction required to effectively shift hepatic metabolism to fatty acid oxidation depends upon induction of pathways, such as AMP- activated kinase (AMPK). We hypothesized that PFOS administration will interfere with CR-induced reduction of hepatic lipids and improve glucose tolerance. Throughout our studies, we found that PFOS increases hepatic lipid content

and decreases the ability of mice to effectively decrease this lipid content upon CR intervention. *In vitro* studies have shown that PFOS interferes with CR when challenged with Metformin and decreases the ability of Metformin to reduce glucose within the media. PFOS decreases the beneficial effects of a calorically restricted diet and interferes with Metformin's glucose lowering effects.



Acknowledgements

This work was supported by National Institute of Health [5R01ES016042-04; 3R01ES016042-2S2; 5K22ES013782-03], and also supported, in part, by Rhode Island IDeA Network of Biomedical Research Excellence grants from the National Center for Research Resources (5P20RR016457-11) and the National Institute for General Medical Science (8P20GM103430-11), components of the National Institutes of Health (NIH). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

Conflict of Interest

There are no conflicts to disclose.

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Figure 1. Adult male mice were administered vehicle (Water, VEH) or perfluorooctane sulfonate (PFOS, 0.1mg/kg/day) and fed ad libitum (AL) or placed on 25% kCal caloric restriction (CR) for approximately 6 weeks. **(A)** Percent change (%) in body weight from day 0 and **(B)** the AUC of percent body weight illustrate that PFOS did not significantly alter CR-induced percent weight at the time of necropsy. In mice that underwent CR, **(C)** Liver weight and **(D)** white adipose tissue (WAT) weight were not statistically different upon PFOS administration compared to vehicle. Groups without a common letter are statistically different ($p<0.05$).

Figure 2. Effect of PFOS and CR on glucose homeostasis. **(A)** Raw glucose tolerance test (GTT) data and **(B)** percent change (%) in glucose compared to time 0. CR significantly improved glucose clearance at 0.5 and 1 hours, which did not occur in mice that underwent CR with PFOS administration. **(C)** Total glucose load of GTT plotted as an area under the curve (AUC). PFOS significantly increased total glucose load in CR groups. **(D)** Raw data of the insulin tolerance test (ITT). The glucose load was not different between groups until 2.5 hours where the two PFOS groups had significantly higher glucose load compared to controls. **(E)** Hepatic mRNA data. Irs-1 and Glut2 expression are significantly decreased in mice that underwent CR upon PFOS administration. Glut2 expression was significantly decreased in ad libitum with PFOS compared to control. **(F)** PFOS did not markedly alter phosphorylated

akt protein levels in these mice. Asterisks (*) represent a statistical difference in treatments groups compared to controls.

Figure 3. PFOS significantly increased hepatic lipid content. **(A)** Liver histopathology. H&E staining of liver tissue illustrates PFOS increase vacuolization in mice that underwent CR compared to control. Hepatic **(B)** free fatty acids (FFA) were statistically decreased upon PFOS administration. **(C)** Hepatic triglycerides were statistically increased upon PFOS administration**.** **(D)** Hepatic mRNA of cyp4a14 and **(E)** ppar-α and downstream targets. Cyp4a14 was statistically induced in mice that underwent CR and PFOS administration compared to control. Groups without a common letter are statistically different.

Figure 4. Hepatic protein expression and quantification. **(A)** Western blots of proteins involved in glucose homeostasis and lipogenesis in mice that underwent CR. **(B)** Western blots of proteins involved in the CR pathway. **(C)** PFOS significantly decreased phosphorylated akt protein levels while significantly increasing pepck protein levels in mice that underwent CR compared to control. **(D)** PFOS significantly decreased p-ampk in mice that underwent CR compared to control. Asterisks (*) represent groups that are statistically significant compared to control.

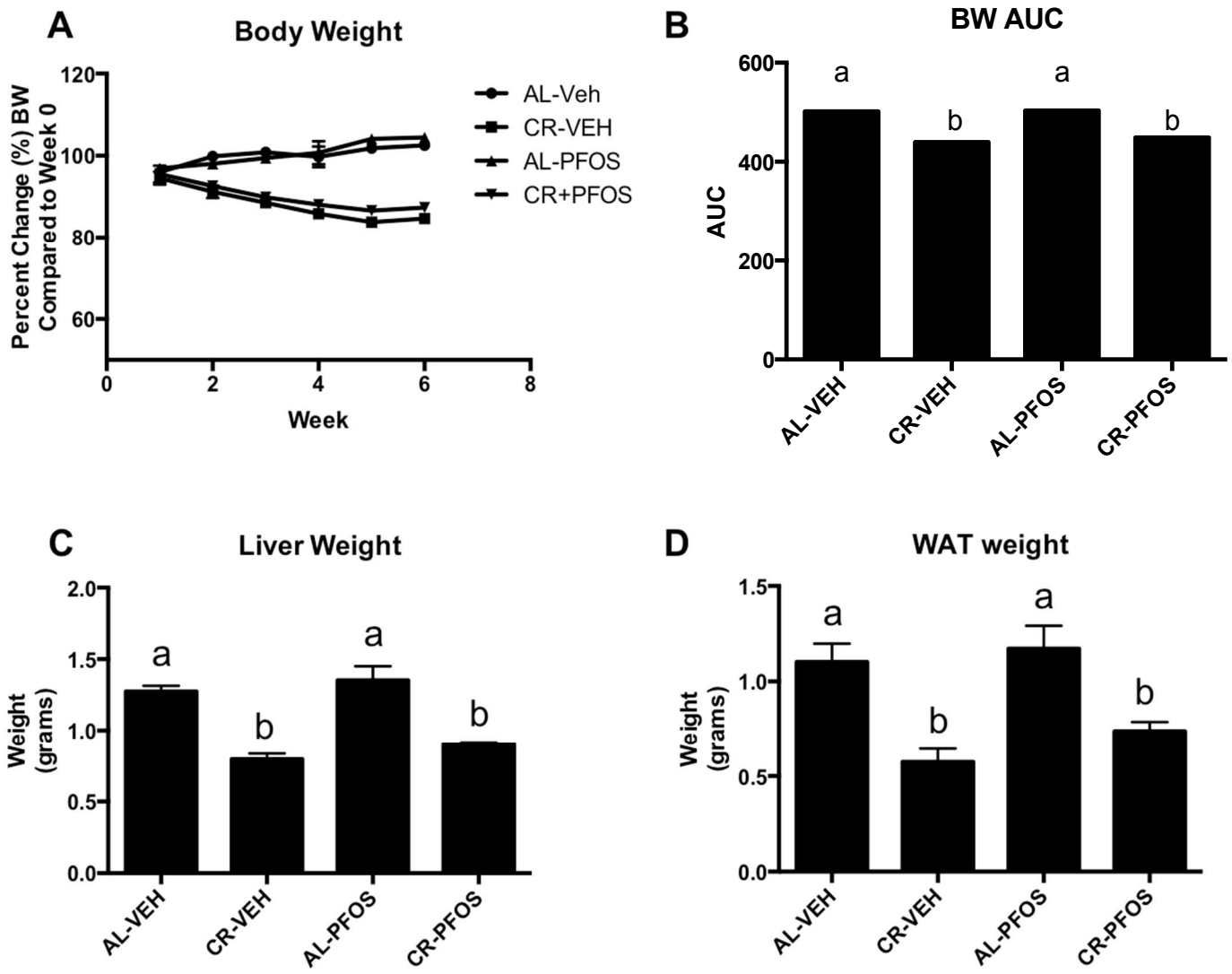
Figure 5. *In vitro* glucose and protein measurements in primary hepatocytes and HepG2 cell lines. **(A)** Media glucose measurement in primary hepatocytes with treatments of 6 hours with glucagon, PFOS (2.5uM), and in combination. PFOS statistically increased glucose in the media of primary hepatocytes. **(B)** Media glucose measurement in HepG2 cells with treatment of 10 hours with Metformin (1mM), PFOS (25 and 100uM), and a combination of Metformin with both concentrations of PFOS. Metformin significantly decreased glucose in the media while PFOS100uM significantly increased glucose. A combination of Metformin and PFOS100uM was significantly increased compared to Metformin alone. *, $p<0.05$, Metformin compared to DMSO; PFOS100um compared to DMSO. #, $p<0.05$, PFOS100uM + Metformin compared to Metformin alone. **(C)** Media glucose measurement in HepG2 cells with treatments of 24 hours with AICAR (100uM), PFOS (0.025uM-25uM) and a combination of AICAR with each PFOS concentration. Both PFOS 25uM and AICAR with PFOS25uM significantly increase glucose in media compared to DMSO control. AICAR with PFOS25uM significantly increases glucose in media compared to AICAR alone. *, $p<0.05$, PFOS25uM and AICAR compared with DMSO control. #, $p<0.05$, AICAR with PFOS25uM compared to AICAR alone. **(D)** Western blot of P-ampk (n=1) of HepG2 cells treated with PFOS (25 and 50uM) and Metformin (1mM). PFOS decreases p-ampk protein expression when co-treated with Metformin, diminishing beneficial effects of Metformin.

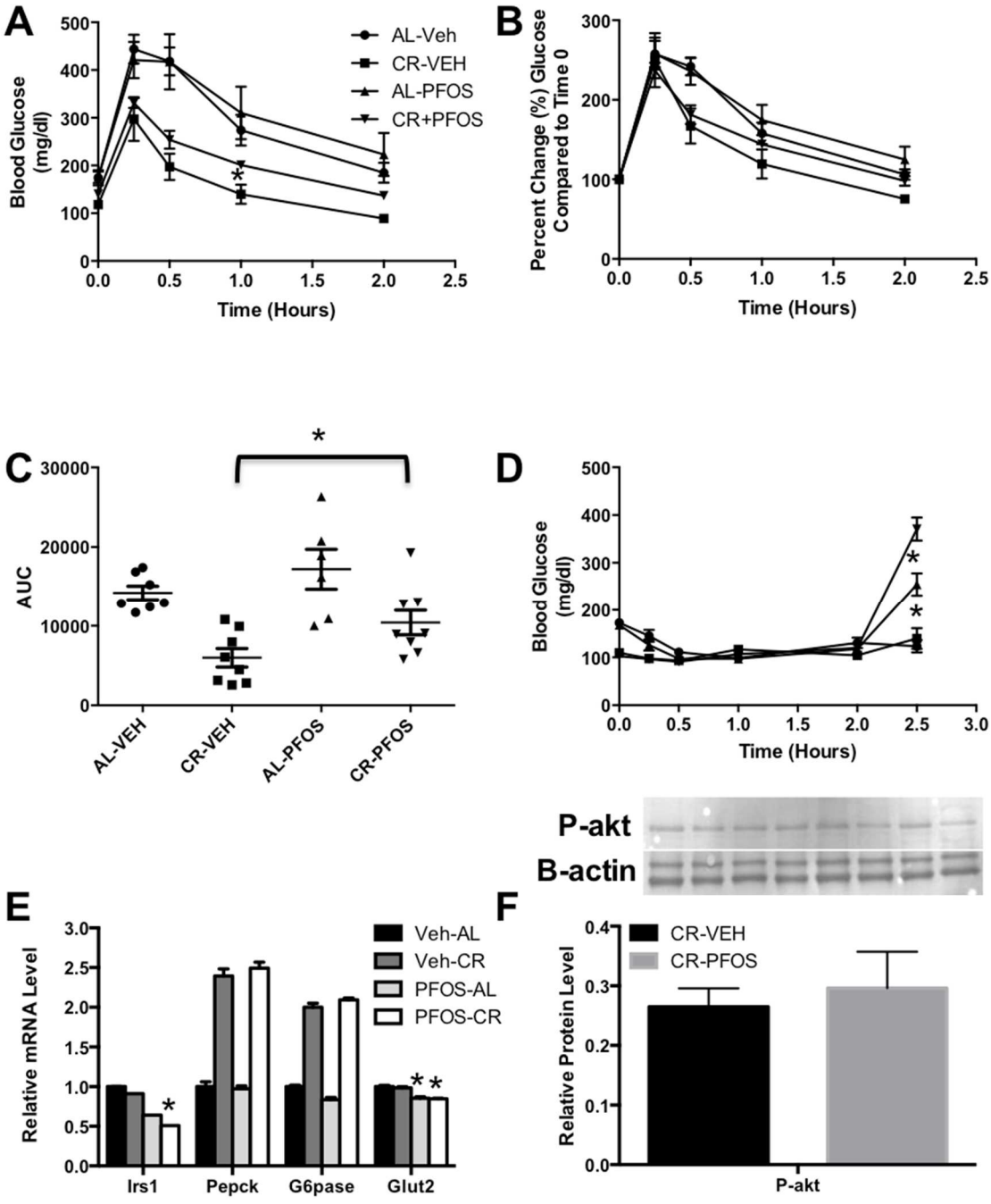
Figure 6. Analysis of glucose homeostasis in mature adipocytes and human adipocytes. 3T3L1 cells were treated with Metformin (1mM), PFOS (50uM) and a combination. **(A)** Media glucose of mature 3T3L1 cells was significantly increased upon PFOS treatment. **(B)** PFOS significantly increased adiponectin levels in mature adipocytes upon PFOS treatment compared to control. **(C)** Human adipocytes were treated with DMSO, Metformin (1mM), PFOS (50uM), and a combination of PFOS with Metformin. PFOS increased the glucose in the media of these cells ($p<0.06$) and significantly decreased glucose when treated in combination with Metformin. Asterisks (*) represent a significant difference compared to control. **(D)** Oil-Red-O staining of the human adipocytes. Mag.10x.

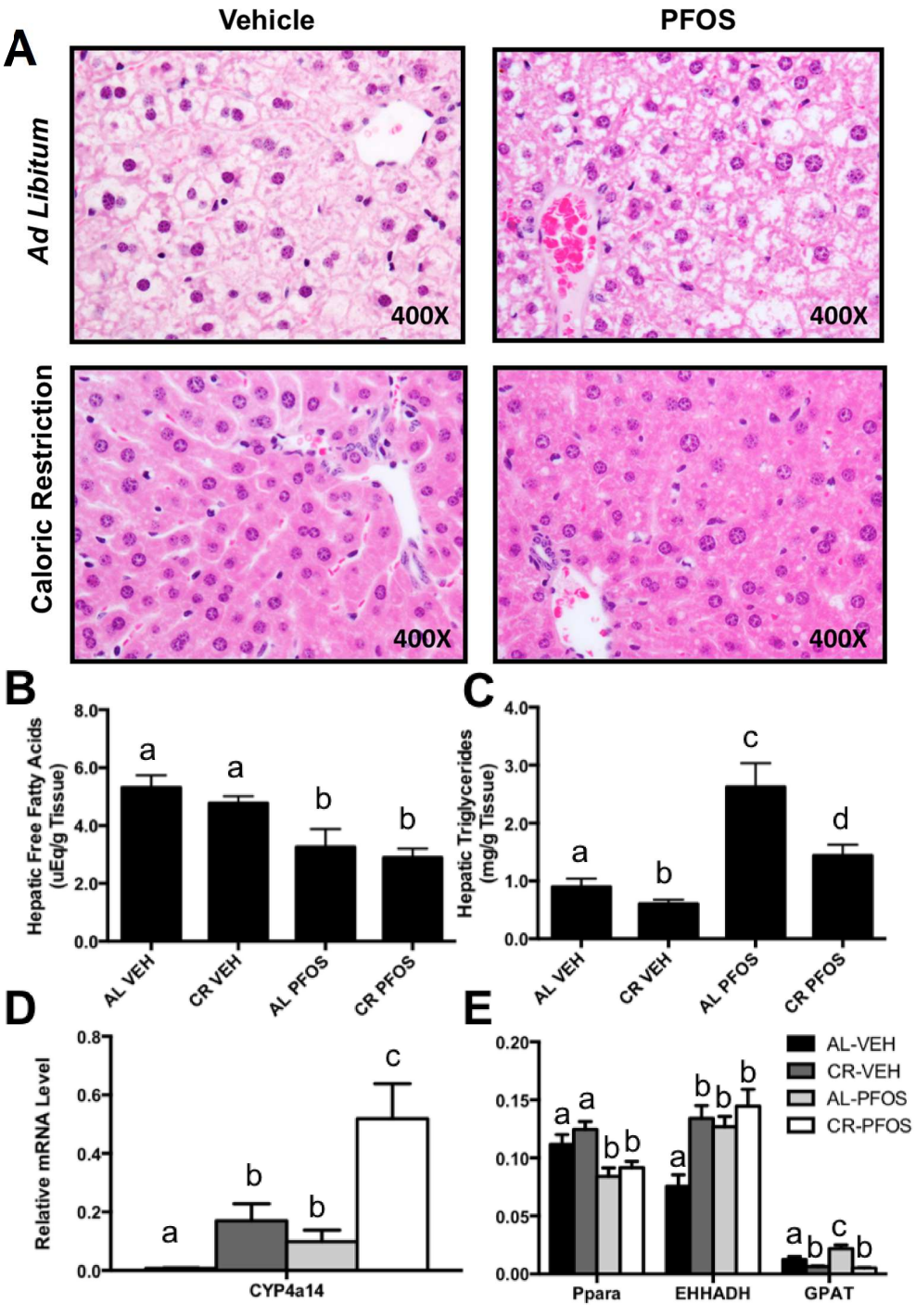
Table 1. Serum Metabolic Measurements. Adiponectin, cholesterol, free fatty acids, glucose and triglycerides measurements of mice after euthanization.

Parameters	Unit	AL-VEH	CR-VEH	AL-PFOS	CR-PFOS
Adiponectin	ng/ml	11007.34±596.02	13674.87±459.70*	13579.57±974.31$	13565.01±668.01#
Cholesterol	mg/dl	120.01±10.92	108.45±14.85	113.95±10.46	98.19±4.64
FFA	µmol/l	0.96±0.15	1.10±0.11	1.15±0.35	1.06±0.10
Glucose	ng/ml	3744.83±155.85	2637.29±210.06*	6189.69±394.14$	4414.46±162.14#,§
Triglycerides	mg/dl	172.08±42.49	130.89±17.00	154.63±17.31	137.50±4.31

Eighteen-week old C57Bl/6 mice underwent treatment for approximately six weeks of either water (VEH) or Perfluorooctanesulfonic acid (PFOS) administration (0.1mg/kg/day) and fed either ad libitum (AL) or underwent 25% kCal caloric restriction (CR). After euthanization, these parameters were assayed and analyzed. *, $p<0.05$, CR-VEH compared to AL-VEH control. $, $p<0.05$, AL-PFOS compared to AL-VEH control. #, $p<0.05$, CR-PFOS compared to AL-VEH control. §, $p<0.05$, CR-PFOS compared to CR-VEH control.


A
Body Weight
Percent Change (%) BW Compared to Week 0
120
100
80
60
0
2
4
6
8
Week
AL-Veh
CR-VEH
AL-PFOS
CR+PFOS
B
BW AUC
AUC
600
400
200
0
a
b
a
b
AL-VEH
CR-VEH
AL-PFOS
CR-PFOS
C
Liver Weight
Weight (grams)
2.0
1.5
1.0
0.5
0.0
a
b
a
b
AL-VEH
CR-VEH
AL-PFOS
CR-PFOS
D
WAT weight
Weight (grams)
1.5
1.0
0.5
0.0
a
b
a
b
AL-VEH
CR-VEH
AL-PFOS
CR-PFOS


A
Blood Glucose (mg/dl)
Time (Hours)
AL-Veh
CR-VEH
AL-PFOS
CR+PFOS
B
Percent Change (%) Glucose Compared to Time 0
Time (Hours)
C
AUC
AL-VEH
CR-VEH
AL-PFOS
CR-PFOS
D
Blood Glucose (mg/dl)
Time (Hours)
P-akt
B-actin
E
Relative mRNA Level
Veh-AL
Veh-CR
PFOS-AL
PFOS-CR
Irs1
Pepck
G6pase
Glut2
F
Relative Protein Level
CR-VEH
CR-PFOS
P-akt


A
Vehicle
PFOS
Ad Libitum
Caloric Restriction
400X
400X
400X
400X
B
Hepatic Free Fatty Acids (uEq/g Tissue)
8.0
6.0
4.0
2.0
0.0
a
a
b
b
AL VEH
CR VEH
AL PFOS
CR PFOS
C
Hepatic Triglycerides (mg/g Tissue)
4.0
3.0
2.0
1.0
0.0
a
b
c
d
AL VEH
CR VEH
AL PFOS
CR PFOS
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a a b b
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GPAT
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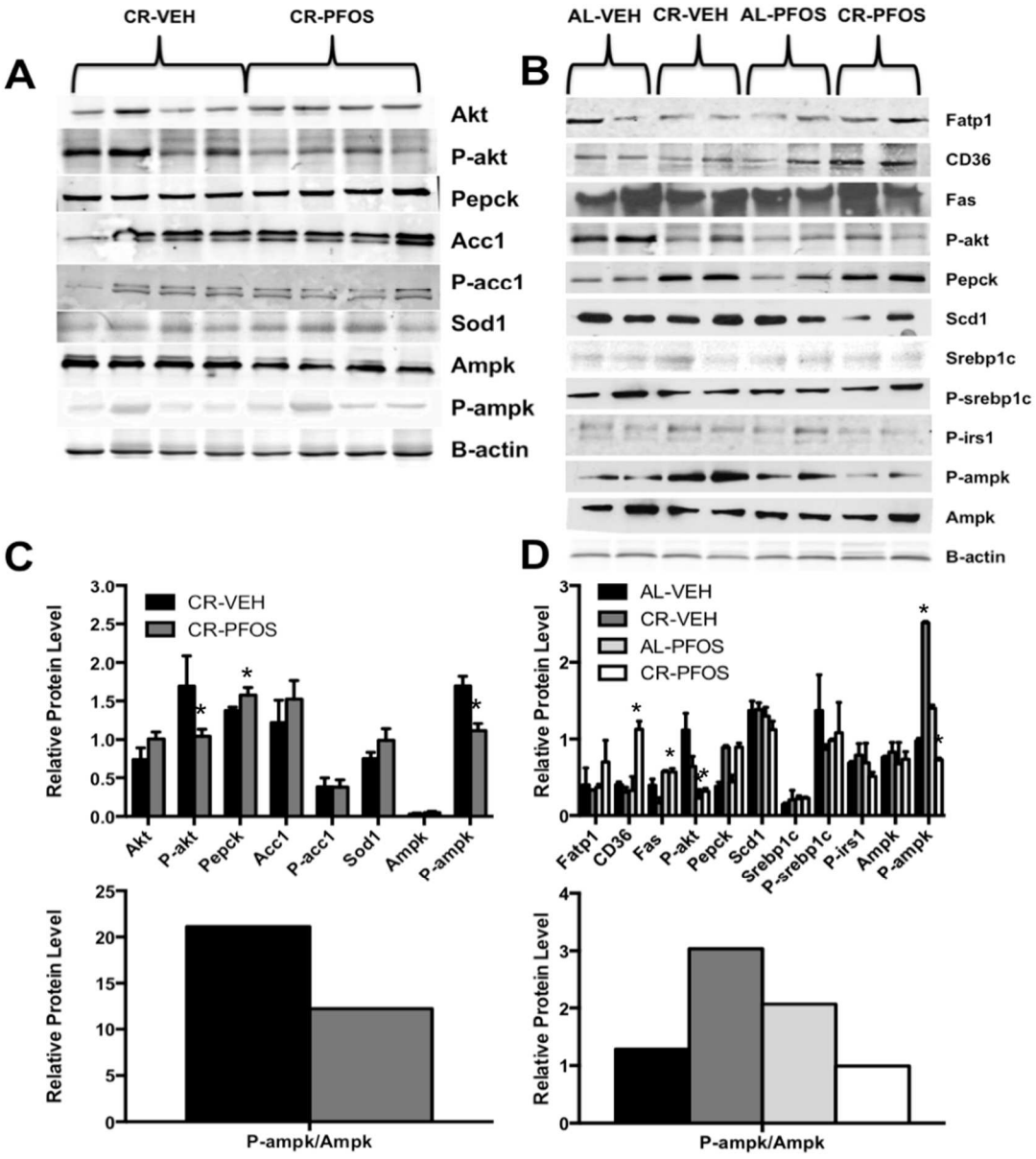
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Fatp1
CD36
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Pepck
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P-irs1
P-ampk
Ampk
B-actin
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Relative Protein Level
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CR-PFOS
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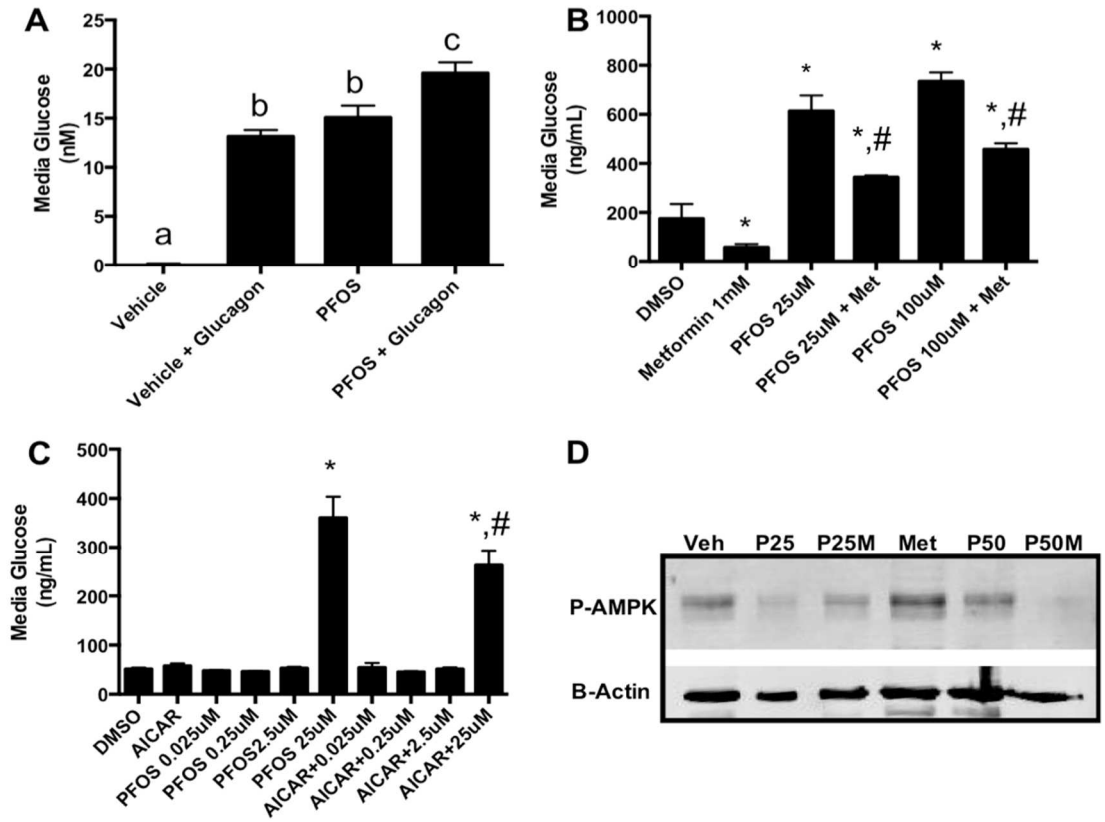
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PFOS 25uM
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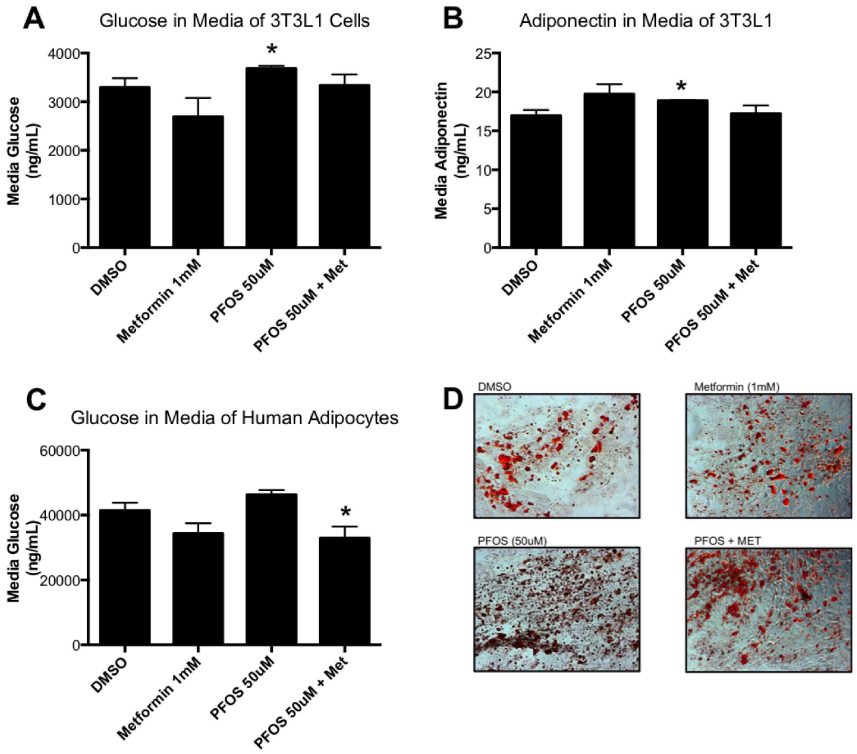
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Chapter 3

MANUSCRIPT II

Short Term PFOA Treatment Does Not Markedly Affect Key Lipogenic and Antioxidant Gene Expression in Adipose Tissue

Deanna M. Salter[1], Jialin Xu[1], Lauren Aleksunes[2], Angela L. Slitt[1]

[1]Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI, [2]Department of Pharmacology and Toxicology, Rutgers University, Piscataway, NJ, USA

RUNNING TITLE: PFOA DOES NOT AFFECT GENE EXPRESSION IN ADIPOSE TISSUE

KEYWORDS: Perfluorooctanoic acid (PFOA), Nrf2, nfe2l2, SREBP1, FAS, ACC1, SCD1, PPAR-alpha

Manuscript in Preparation

TO WHOM PROOFS SHOULD BE SENT:

Angela L. Slitt, Ph.D.

Department of Biomedical and Pharmaceutical Sciences

University of Rhode Island

41 Lower College Road

Kingston, RI 02881

Phone: (401) 874-5939

Fax: (401) 874-5048

E-mail: aslitt@etal.uri.edu

ABSTRACT: Perfluorooctanoic acid (PFOA) is a perfluorinated carboxylic acid commonly found in the environment. According to the EPA, low levels of PFOA are detectable in the environment and in the blood of the general United States population. PFOA administrations cause liver and developmental toxicity in rodents and is found in serum and tissues of wildlife and humans worldwide. PFOA is a potent activator of peroxisome proliferator receptors (Ppars) contributing to oxidative stress and fatty acid oxidation pathways in hepatocytes and possibly in adipose tissue (ppar-γ). Since PFOA is extremely ubiquitous and persistent, our hypothesis is that it would increase gene expression in adipose tissue of mice in regard to fatty acid oxidation, lipid synthesis and antioxidant response. Adult male mice were treated with 1.0 or 3.0 mg PFOA/kg in corn oil for 7 days. Adipose tissue was collected and total RNA was isolated. Analysis of mRNA was completed by quantitative PCR. mRNA expression between control and PFOA treated mice was similar. Our results indicate PFOA has no affect on adipose tissue of mice after a 7-day treatment. Literature is lacking information regarding PFOA effects in adipose tissue.

INTRODUCTION

Perfluorooctanoic acid (PFOA) is a synthetic surfactant and is expressed in sera throughout the world of humans and animals (EPA, 2009a; and Hines, *et al.* 2009). PFOA is used in the manufacturing process to make fluoropolymers with specific properties for various industrial applications including fire resistance, water, oil, and grease repellency as well as providing non-stick surfaces for cookware (EPA, 2009a). PFOA is also produced from the breakdown of fluorinated telomers such as grease, soil, stain and water resistance (EPA, 2009a). The Environment Protection Agency (EPA) is investigating PFOA as a possible toxin to humans due to the presence of an closely related PFC, perfluorooctyl sulfonates (PFOS), in the blood of the general United States population which raised concern for its persistence in the environment, bioaccumulation and toxicity in the late 1990's (EPA, 2009a). Manufacturing of PFOS ceased and the EPA started investigating similar chemicals that might elicit comparable apprehensions (EPA, 2009a). PFOA has been investigated since the 1990's and is known to elicit liver effects (hepatomegaly), development toxicant, and carcinogen in rodent models and found in serum and tissues of wildlife and humans worldwide with an average half-life of 3.5 years in humans (EPA, 2009a; Hines, *et al.* 2009; Klaunig, *et al.* 2003; Tardiff*, et al.* 2009). PFOA stimulates similar effects to peroxisome proliferating chemicals (PPC), which have been shown to provoke liver cancer due to the induction of peroxisome proliferater-activated receptor alpha (PPAR-alpha) (Rosen, *et al.* 2008b). Calafat et al suggests that men have

higher serum concentrations of perfluoroalkyl acid's (PFAA) than women, and the younger population has higher concentrations than the older population (Calafat, *et al.* 2007). In Danish men, Joensen et al found that higher PFAA concentration is associated with fewer normal spermatozoa (Joensen, *et al.* 2009). PFOA can cross the placental membrane and has been found in umbilical cord blood in levels that are negatively associated with newborn weight (Apelberg, *et al.* 2007). Human studies of PFOA are limited, however, trace amounts of PFOA have been detected in seminal plasma samples, cord blood, and low levels in urine associated with high levels of biliary reabsorption rates, (Apelberg, *et al.* 2007; Harada, *et al.* 2007; and Joensen, *et al.* 2009). Maestri et al found 0.1ng/g PFOA in all human tissue except for adipose tissue, where it was 0.2ng/g PFOA in the general population with very low exposure of PFOA (Maestri, *et al.* 2006).

Since there is cross-talk among the liver tissue with peripheral tissues, if there are effects within the liver, then these effects may also affect adipose tissue. Some interconnecting enzymes include sterol regulatory element binding protein 1 (SREBP1) regulates which genes involved in lipid biosynthesis including fatty acid synthetase (FAS) and acetyl co-A carboxylase (ACC1) involved in de novo lipogenesis, diacylglycerol acetyltransferase (DGAT), glycerol-3-phosphate acyltransferase (GPAT) and lipoprotein lipase (LPL) involved in the reesterification of fatty acids, and stearoyl co-A desaturase (SCD1) involved in fatty acid desaturation and elongation (Hagen, *et al.* 2010). Peroxisome proliferator-activated receptor alpha (PPAR-alpha) is found

predominantly in the liver and is involved in lipid and lipoprotein metabolism reducing triglyceride levels as well as maintaining energy homeostasis regulating obesity (Tyagi, *et al.* 2011). PPAR-gamma targets LPL and is involved in lipid metabolism and lipid uptake into the adipocytes and is involved in adipocyte differentiation and hypertrophy (Tyagi, *et al.* 2011). Ppar-y is highly expressed within adipose tissue and may be an unrecognized target for various industrial or environmental chemicals within society, such as PFOA (Malone and Waxman, 1999).

Nuclear factor-2 p45 related factor 2 (NRF2) is a basic leucine-zipper transcription factor that serves as a cellular detoxicant by positively regulating over 200 cytoprotective genes that defend against stressors, such as toxic chemicals and radiation (Klaassen, *et al.* 2010; Niture, *et al.* 2011; Sykiotis, *et al.* 2011; Young-Sam, *et al.* 2006). The genes include phase II drug metabolizing enzymes and antioxidant proteins (Young-Sam, *et al.* 2006). NRF2 is sequestered in the cytoplasm via Kelch-like ECH-associated protein (Keap1) (Giudice, *et al.* 2010; Young-Sam, *et al.* 2006). Upon activation by stressors, NRF2 is released from Keap1 and translocates into the nucleus, binding to antioxidant response elements (ARE)/electrophile response elements (EpREs) in the promoter region and eliciting activation of cytoprotective genes (Giudice, *et al.* 2010; Niture, *et al.* 2011; Young-Sam, *et al.* 2006). Keap1 travels into the nucleus binding to NRF2 dissociating it from ARE and the complex is exported to the cytoplasm via the nuclear export sequence of keap1 (Giudice, *et al.* 2010). Natural and synthetic

chemopreventive agents induce antioxidative and carcinogen-detoxification enzymes via the NRF2/keap1 pathway (Giudice, *et al.* 2010). Nrf2 controls the basal and inducible expression of mouse and human genes, such as NQO1, GCLC, and GSTA1 (Klaassen, *et al.* 2010; Ramos-Gomez, *et al.* 2001). Oxidative stress is a stressor that activates nrf2/keap1 pathway and has been linked to insulin resistance due to the increase in mitochondrial reactive oxidative species (ROS) (Yu, *et al.* 2012). ROS oxidizes cysteines of the keap1 dissociating it from nrf2 allowing the translocation of nrf2 to the nucleus (Ray, *et al.* 2012). When Nrf2 is activated by known activators on a high-fat diet, the increase in adipose tissue mass, hepatic lipid accumulation and body-weight was attenuated (Yu, *et al.* 2012). However, other studies have found conflicting data. Pi et al. suggest that Nrf2 knockout mice have decreased fat mass with more small adipocytes and portray diet-induced obesity resistance. In this same study, Nrf2 knock out mouse embryonic fibroblasts (MEF's) displayed decreased adipogenesis. In the 3T3-LI cell line and human subcutaneous preadipocytes, Pi et al demonstrated adipocyte differentiation inhibition with shRNA-mediated nrf2 knockdown possibly attributing to the association with, and subsequent decrease of, PPAR-gamma expression (Pi, *et al.* 2010). Previous data from our lab indicate that in nrf2 deficiency in $Lep^{ob/ob}$ (leptin-deficient-obese) mice induced IR and dyslipidemia while decreasing white adipose tissue mass and preventing hepatic lipid accumulation indicating a dual role for Nrf2 in metabolic dysregulation (Xu *et al.,* 2014).

In this study, adult male mice were treated with 1.0 mg PFOA/kg or 3.0 mg PFOA/kg in corn oil for 7 days. We hypothesized that PFOA would alter gene expression in adipose tissue of mice in regard to fatty acid oxidation, lipid synthesis and antioxidant response.

MATERIALS AND METHODS

Animals and treatment paradigm

Animal treatment and PFOA dosing we previously used to investigate effects PFOA in kidney (Aleksunes, *et al.* 2013). Briefly, adult male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, WA) and fed a normal rodent chow diet (Aleksunes *et al.*, 2013). The mice were split into three groups (n=6); control, low dose PFOS (1.0 mg/kg) and high dose PFOS (3.0 mg/kg). Perfluorooctanoic acid (PFOA) (77262, Sigma-Aldrich, St. Louis, MO) was administered once per day at a dose volume of 5ml/kg for seven days. For *1 mg/kg dose,* 2 mg PFOA was dissolved in 1 ml deionized (DI) water. This stock was vortexed and 9 ml of DI water was added to make a 2mg/10 ml solution that was filter sterilized. For *3mg/kg dose,* 6 mg PFOA was dissolved in 1 ml DI water and vortexed. Then 9 ml DI water was added to make a 6mg/10 ml solution, which was filter sterilized. Organs and tissues were collected and snap frozen and stored in -80 until RNA analysis.

PCR Assay

Total RNA was isolated from the collected adipose tissue by phenol-chloroform extraction with 1.2mL Trizol (Invitrogen, Camarillo, CA)/100mg tissue according to the manufacturer's protocol. RNA concentration were determined by measuring UV absorbance at 260 nm using NanoDrop™. The total RNA samples were stored at -80°C until further use for analysis. 1 µg of total RNA was converted to cDNA, and mRNA levels were quantified by

quantitative real-time PCR using a Roche LightCycler 480 System (Roche Applied Science, Mannheim, Germany). Genes measured were Acc-1, Scd-1, Lpl, Ppar-γ, Srebp-1, Nrf2, Nqo-1, Gclc, Irs-1, Keap-1, and Ahr. SYBR green reagent was used, and relative target gene expression was normalized to GAPDH or 36B4 mRNA.

Statistical Analysis

Statistical analyses of differences were performed by Student's t test. $P < 0.05$ was considered statistically significant. Unless otherwise stated, all data were presented as mean ± SE of five animals.

RESULTS

PFOA treatment did not affect body weight, however, did increase liver weight.

PFOA has been suggested to induce body weight gain in mice, however, in our study, the mice did not have and increased average body weight compared to the vehicle in either 1.0 mg/kg or 3.0 mg/kg PFOA which is illustrated in Figure 1. It is suggested that mice with PFOA administration have increased liver weights due to an accumulation of hepatic triglycerides. In our study, there was a statistical increase that is dose-dependent. The white adipose tissue weight was unchanged between groups. Kidney tissue weights were increased with a treatment of 1.0 mg/kg PFOA compared to vehicle, but not in the 3.0 mg/kg PFOA treatment.

PFOA treatment did not affect lipid synthesis and accumulation gene expression in white adipose tissue.

Srebp1c is the regulator of lipid synthesis and acts on its target genes, Scd1 and Acc1. As shown in Figure 2, Srebp1c mRNA expression was significantly increased in both treatment groups, while Scd1 and Acc1 were not significantly different among treatment groups; however, Scd1 possesses an increasing PFOA dose-dependent trend. Ppar-α is involved in fatty acid oxidation with a downstream target of Cyp4a14. PFOA is suggested to be an agonist for Ppar-α, however, in white adipose tissue with 1.0 and 3.0mg/kg PFOA for 7 days, Ppar-α gene expression was not statistically different than

that of vehicle. PFOA treatment did not significantly alter Cyp4a14 compared to vehicle. Lpl codes for lipoprotein lipase which is involved in the breakdown of triglycerides to free fatty acids that can be uptaken into tissues, mainly skeletal muscle, adipose tissue and cardiac muscle. PFOA did not significantly alter Lpl gene expression in either treatment group compared to vehicle. PPAR-γ is involved in adipocyte differentiation and was not significantly altered with PFOA administration.

PFOA treatment did not affect oxidative stress gene expression in adipose tissue

Oxidative stress mRNA expression is illustrated in Figure 3. The transcription factor, Nrf2, is sequestered in the cytosol by Keap1 until phosphorylation where Nrf2 translocates to the nucleus activating the initiation of transcription of its target genes, Ahr and Gsta1. Nrf2 is significantly increased with 1.0 mg/kg PFOA treatment compared to vehicle, however, 3.0 mg/kg PFOA treatment is not. Downstream targets of Nrf2 were not significantly different among treatment groups.

DISCUSSION

The purpose of this study was to evaluate the effects of PFOA exposure on adipose tissue. Overall, the findings suggest PFOA does not markedly affect adipocyte differentiation, lipid synthesis and accumulation, or oxidative stress response in adipocytes.

PFOA is structurally similar to a fatty acid and acts similarly as it activates nuclear receptors, binds to transporters and carrier proteins and interacts with membranes (Post, *et al.* 2012). PFOA activates PPAR-alpha and other nuclear receptors (Post, *et al.* 2012). SREBP-1 was significantly increased in the 3mg/kg PFOA treated mice. SREBP-1 regulates genes involved in lipid biosynthesis such as FAS, SCD-1, ACC-1, and LPL (Hagen, *et al. 2010*). SCD-1 and ACC-1 remained unchanged in all treatment groups, however, elicit increasing PFOA-dependent trends. A recent review suggests that effects such as obesity and metabolic changes are not evident until long after PFOA has been eliminated from the body, which may be a factor that our findings are not statistical, however show slight trends (Post, *et al.* 2012). Also, it is suggested that prenatal PFOA exposure is associated with the increased risk of obesity and metabolic hormone differences in 20-year old women (Post, *et al.* 2012).

The increase in body weight of mice in a PFOA-dependent manner could be due to increases of triglycerides, phospholipids, and cholesterol accumulation in the liver, which induced fatty liver in mice caused by PFOA administration (Post, *et al.* 2012).

Other studies report findings primarily based on liver tissue. Steenland et al suggest there is a significant, positive correlation of PFOA and cholesterol levels (Steenland, *et al.* 2010). Although the mode of action is not well understood, PFOA's action is suggested to be part PPAR-alpha-dependent and part PPAR-alpha-independent. PPAR-alpha independent is thought to be because the branched isomers of PFOA increased liver weight but was less effective in activating PPAR-alpha (Post, *et al.* 2012). Studies suggest that PFOA increases body weight of mice even at low doses of 0.01mg/kg-0.30mg/kg (Wan *et al.,* 2012; Yan *et al.,* 2014). Also, in PPAR-alpha-null mice, PFOA is also hepatotoxic, contributing to the PPAR-alpha-independent mechanism of PFOA (Post, *et al.* 2012).

The EPA is investigating PFOA for possible toxic effects. Humans have trace amounts of PFOA in serum and tissues (EPA, 2009a). The endpoints of PFOA in humans and experimental animals are usually parallel; however, differ in lipid metabolism outcome (Post, *et al.* 2012). Literature is abundant with effects of PFOA in the liver but lacks data in adipose tissue. This preliminary study is one of the first to explore the mechanism of PFOA in adipose tissue that may be attributable to its toxic effects.

Acknowledgements

This work was supported by National Institute of Health [5R01ES016042-04; 3R01ES016042-2S2; 5K22ES013782-03], and also supported, in part, by Rhode Island IDeA Network of Biomedical Research Excellence grants from the National Center for Research Resources (5P20RR016457-11) and the National Institute for General Medical Science (8P20GM103430-11), components of the National Institutes of Health (NIH). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of Interest

There are no conflicts to disclose.

FOOTNOTES

This work was presented, in part, at the Annual Society of Toxicology meeting held March 14, 2012, in San Francisco, California.

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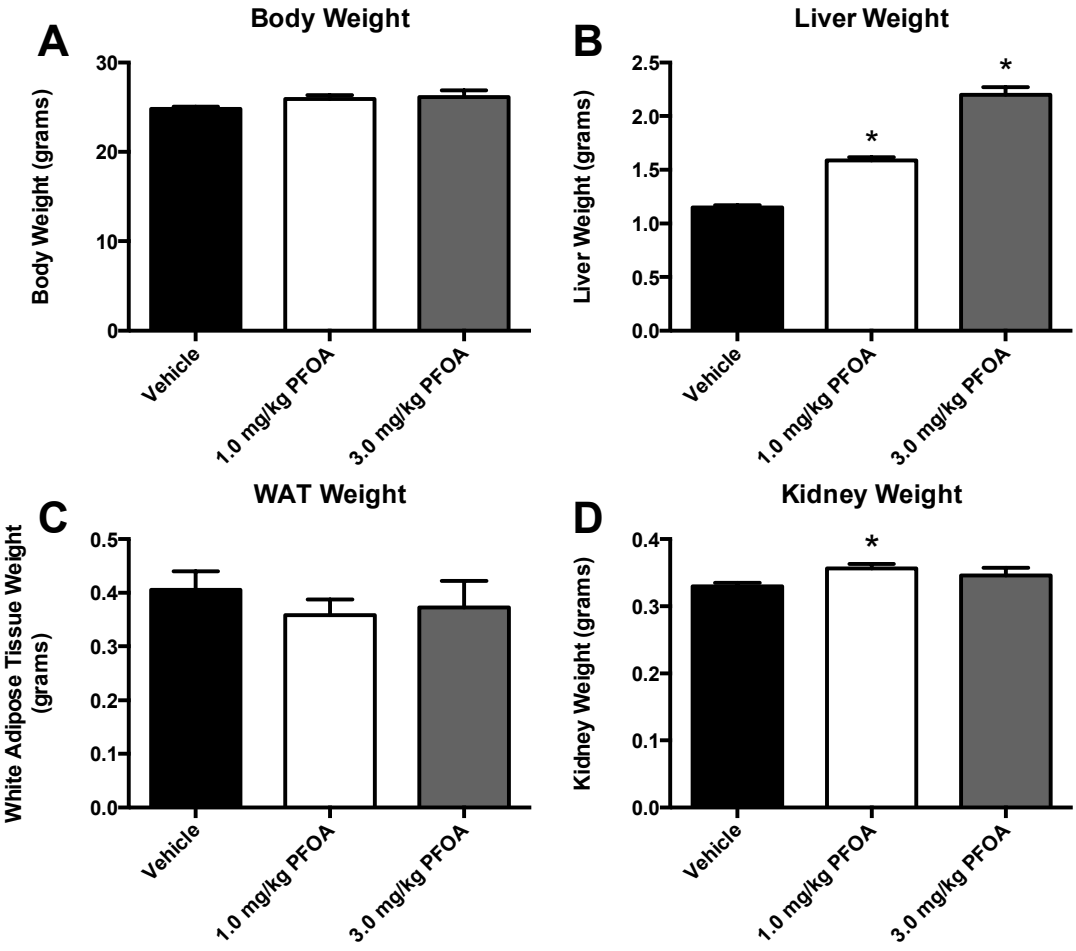
Figure 1. Overall body and organ weights after PFOA treatment. **(A)** Average body weight measurements after PFOA administration. **(B)** Average liver weight measurements after PFOA administration. *, $p<0.05$, PFOA treatment groups are both statistically higher than the vehicle liver weights. **(C)** Average white adipose tissue weight after PFOA administration. **(D)** Average kidney weight measurement after PFOA administration. *, $p<0.05$, 1mg/kg PFOA administration statistically increased average kidney weights compared to vehicle kidney weights.

Figure 2. Fatty acid oxidation and lipid synthesis gene expression in WAT after PFOA administration. There was no alteration in fatty acid oxidation genes (Cyp4a14 and Ppar-a). PFOA administration significantly increased Srebp1c mRNA expression, but did not affect the expression of downstream genes (e.g. Acc-1, Lpl, and Scd-1). Ppar-γ mRNA expression was similar between groups with no statistical differences. *, $p<0.05$, both PFOA treatment groups significantly increased srebp1c gene expression compared to vehicle.

Figure 3. Oxidative stress target gene expression in WAT after PFOA administration. PFOA administration (1 mg/kg) increased nrf2 mRNA expression in WAT. Downstream Nrf2-target genes (e.g. Nqo1, Gclc, Keap1, Gsta-1, and AhR) did not show statistical differences between groups.

*,$p<0.05$, nrf2 gene expression was statistically higher in 1mg/kg PFOA treatment group compared to vehicle.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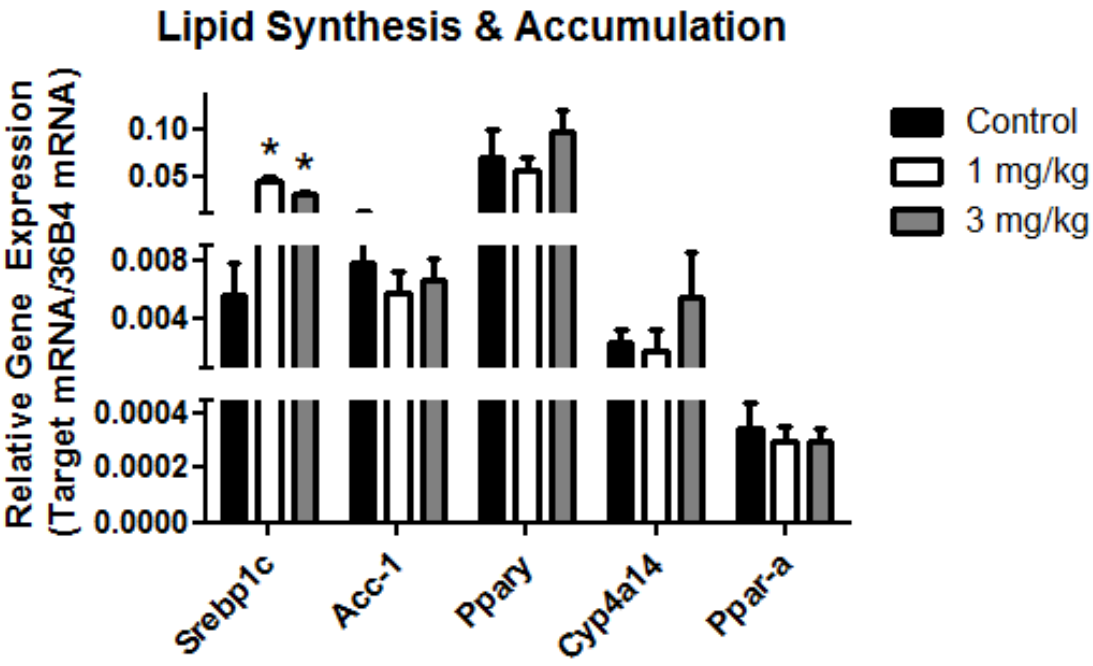
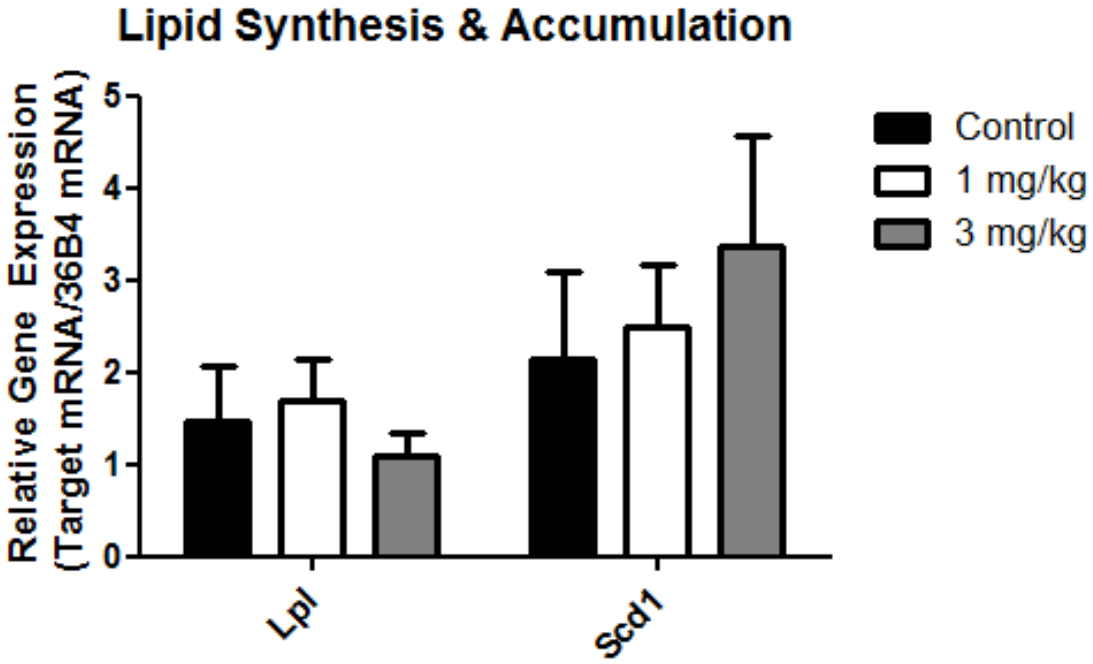
1.0 mg/kg PFOA
3.0 mg/kg PFOA


Lipid Synthesis & Accumulation
Relative Gene Expression
(Target mRNA/36B4 mRNA)
0.10
0.05
0.008
0.004
0.0004
0.0002
0.0000
*
*
Srebp1c
Acc-1
Ppary
Cyp4a14
Ppar-a
Control
1 mg/kg
3 mg/kg


Lipid Synthesis & Accumulation
Relative Gene Expression
(Target mRNA/36B4 mRNA)
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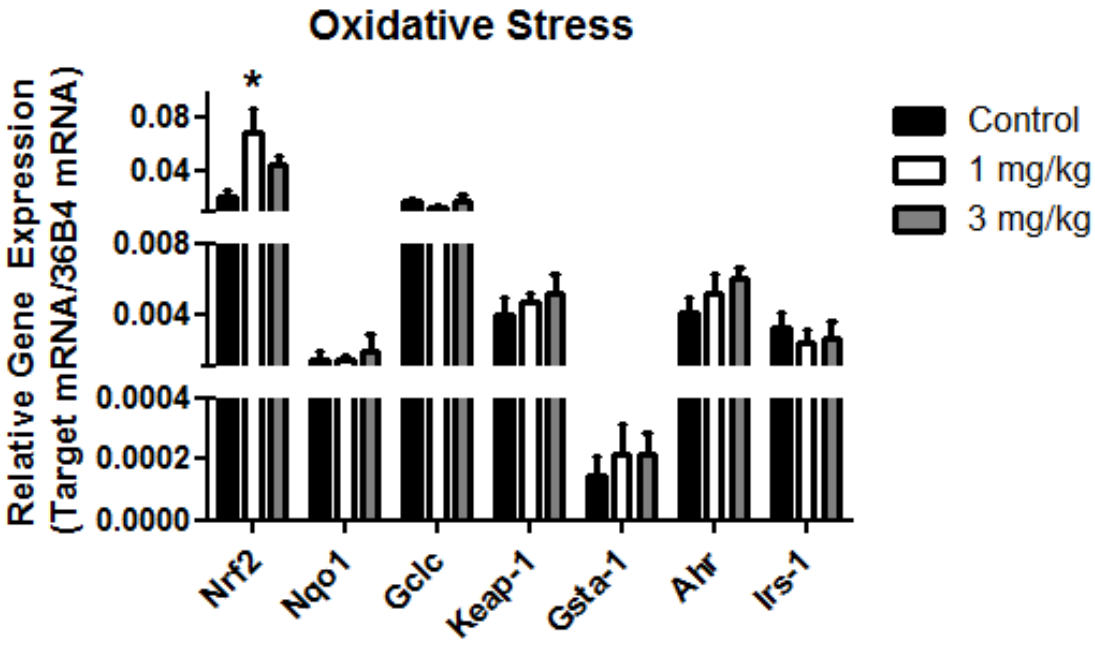
4
3
2
1
0
Lpl
Scd1
Control
1 mg/kg
3 mg/kg


Oxidative Stress
Relative Gene Expression
(Target mRNA/36B4 mRNA)
0.08
0.04
0.008
0.004
0.0004
0.0002
0.0000
*
Nrf2
Nqo1
Gclc
Keap-1
Gsta-1
Ahr
Irs-1
Control
1 mg/kg
3 mg/kg

APPENDICES

Introduction

Oleanolic Acid Decreased Hepatic Cholesterol Synthesis. This appendix material reflects a study that I helped conduct during my graduate studies. We were not able to find a mechanism to describe the observed phenotype and the project was a minor focus of my thesis work. I performed tissue analysis, gene expression, and the western studies. The overall conclusion of this study was that Oleanolic acid decreased hepatic cholesterol synthesis, but the mechanism by which it does so was not uncovered. The appendix material provides key data for the study.

Introduction

Oleanolic acid (OA) is a pentacyclic triterpenic acid found in many fruits in vegetables as secondary plant products and exists as the free acid form eliciting many beneficial nutriceutical properties protecting against hepatotoxicants (Prades, 2011; de Melo, 2010; Reisman, 2010). OA has been used in Chinese medicine for the treatment of liver disorders protecting against oxidative and electrophilic stress and activates the nuclear factor erythroid 2-related factor 2 (Nrf2)/ kelch-like ECH-associated protein-1 (Keap1) pathway inducing expression of cytoprotectant genes (Resiman, 2010). OA is bioactively present in olive leaves, mistletoe sprouts, grape, clove, and pomegranate flowers (de Melo, 2010). Pharmacological and biochemical advantages include anti-inflammatory (most attributable to the suppression of the inducible nitric oxide synthethase gene), anti-hypertensive (OA attenuated endothelial dysfunction), anti-diabetic, anti-viral, anti-hyperlipidemic (inhibition of the ACAT gene which controls enzymes that regulate plasma fatty acid metabolism) and hypoglycemic effects which could be attributable to the antioxidant effect (de Melo, 2010). Pentacyclic triterpenes also show significant suppression of tumor genesis and inhibit tumor formation (Prades, 2011). OA has exhibited agonistic activity of TGR5, a G-protein coupled receptor involved in energy metabolism and is a potential drug target candidate due to its suppression of insulin resistance and anti-obesity effects (Prades, 2011).

OA administration significantly increased expression of nrf2 and its target genes in wild-type mice including nqo-1, gclc, and ho-1, however, not in nrf2-null mice (Reisman, 2010). OA pretreatment reduces acetaminophen hepatotoxicity in nrf2-null mice and more significantly in wild-type mice (Reisman, 2010).

Butylated hydroxyanisole (BHA; 3-*tert*-butyl 4-hydroxyanisole) is a synthetic phenolic compound that has been suggested to be a strong inducer of Nrf2 and two of its downstream targets; Nqo-1 and HO-1 in rat hepatocytes (Keum *et al.,* 2006). BHA has been shown to induce glutathione-S-transferase (GST) and Nqo-1 activities indicating a cytoprotective mechanism possibly through Nrf2 (Hayes *et al.,* 2000).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a basic leucine-zipper transcription factor that serves as a cellular detoxicant by endogenously positively regulating over two hundred cytoprotective genes that defend against stressors, such as toxic chemicals and radiation (Niture, 2011; Sykiotis, 2011; Klaassen, 2010; Reisman, 2010; Wang, 2010; Young-Sam, 2006). The genes include phase II drug metabolizing enzymes and antioxidant proteins (Young-Sam, 2006). Kelch-like ECH-associated protein 1 (Keap1) acts as a tether, sequestering nrf2 in the cytoplasm, at low oxidative and electrophilic stress level conditions (Reisman, 2010; Giudice, 2010; Young-Sam, 2006). In the cytoplasm under low oxidative and electrophilic stress, keap1 is an adapter of Cullin (cul) 3-based E3 ligase which promotes nrf2 degradation (Reisman, 2010). Cul3 acts as a scaffold protein regulating transcription factor turnover and consequently facilitates ubiquitination for protein degradation (Resiman, 2010). Upon oxidative stress, nrf2 translocates into the nucleus, heterodimerizes with a musculo-aponeurotic fibrosarcoma (Maf) protein binding with antioxidant-response elements (AREs)/ electrophile response elements (EpREs) upstream at the promoter of cytoprotectant genes eliciting cellular defense mechanisms restoring homeostasis between oxidants and anti-oxidants (Niture, 2011; Resiman, 2010; Wang, 2010; Giudice, 2010; Young-Sam, 2006). Keap1 travels into the nucleus binding to NRF2 dissociating it from ARE and the complex is exported to the cytoplasm via the nuclear export sequence of keap1 (Giudice, 2010). Natural and synthetic chemopreventive agents induce antioxidative and carcinogen-detoxification

enzymes via the Nrf2/keap1 pathway (Giudice, 2010). Nrf2-null mice exhibit an enhanced susceptibility to hepatic injury (Reisman, 2010; Klaassen, 2010). Nrf2 controls the basal and inducible expression of mouse and human genes, such as NQO1, HO-1, GCLs, EH-1, GSTs, SRXN-1, MRPs, BSEP, CES, SOD1 (Klaassen, 2010; Ramos-Gomez, et al., 2001b).

Hypercholesterolemia:

Cholesterol is metabolized in two pathways; the classic and the alternative (Tiangang, 2009). CYP7A1is the rate limiting enzyme in the classic pathway converting cholesterol to 7-α-hydroxysterol which gets further converted to cholic acid and chenodeoxycholic acid and further dehydroxylated to produce secondary bile acids, deoxycholic acid and lithocholic acid (Tiangang, 2009). CYP27A1 initiates the alternative pathway converting cholesterol to 27-hydroxycholesterol which is further hydroxylated by oxysterol 7α-hydroxylase via CYP7B1 (Tiangang, 2009). Among other reasons, cholelithiasis can arise from a decrease in CYP7A1 expression leading to less bile acid synthesis and more cholesterol build up in the bile creating hard substances, or, gallstones (Biddinger, 2008). Biliary cholesterol and dietary cholesterol diverge to create luminal cholesterol (Tiangang, 2008). Luminal cholesterol can be converted to bile acids through CYP7A1 by 7-α-dehyroxylase or it can be converted to steroids by CYP11A1 (Tiangang, 2008). Before the cholesterol can be engulfed into the eneterocytes, it needs to unite with bile acids to form micellar cholesterol (Duan, 2007). The bile

micelle is engulfed into the enterocyte through the apical membrane NPC1L1 (Neimann-Pick C1-like protein 1) transporter (Duan, 2007). Most of the free cholesterol is subsequently esterified through ACAT (acetyl-Coenzyme A acetyltransferase) while some is taken back to the liver by the MTP/CM (microsomal triglyceride transfer protein/chylomicron) pathway. The minority of cholesterol is excreted through the apical sterol export pump, ABCG5/ABCG8 (ATP-binding cassette transporters) heterodimer, from the enterocyte into the intestinal lumen and from the hepatocytes into the bile flow (Refer to Figure 2) (Rudkowska, 2008). The ABC superfamily of active transporters includes the protein G5 (ABCG5) and protein G8 (ABCG8) which are cholesterol half transporters and come together to form a heterodimer that functions in the transfer of sterols (Rudkowska, 2008) from enterocytes into the intestinal lumen and from hepatocytes into bile for biliary secretion (Ikeda, 2009; Santosa, 2007; Duan, 2006). Expression of this heterodimer promotes the efflux of cholesterol into the lumen thereby reducing cholesterol absorption and consequently resulting in hepatic cholesterol synthesis (Santosa, 2007). Phytosterols are also taken up by the NCP1L1 transporter into the enterocyte, however, are not esterified and are either transported back into the lumen by ABCG5/ABCG8 or go through the ABC1A1 to HDLs and into the periphery (Duan, 2006). Polymorphisms in the genes that encode these transporters have the potential to lead to sitosterolemia, hypercholesterolemia (Santosa, 2007) and gallstone formation (Rudkowska, 2008) by altering their efficacy (Santosa, 2007).

OA significantly decreased total cholesterol when given a HFD and OA compared to the HFD alone (de Melo, 2010).

Study Hypothesis

The purpose of this study was to demonstrate that an nrf2 activator, oleanolic acid, decreased hepatic cholesterol synthesis.

Methods

Animals and Treatments: Adult C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in a temperature-, light-, and humidity-controlled environment in cages with corn-cob bedding. The C57BL/6 mice were pair-wise fed i) standard diet (LM-485 Mouse/rat sterilizable diet, Harlan Laboratories, Madison, WI) , ii) lithogenic diet (LD, 15% fat, 1.25% CH, 0.5% sodium cholate, TD03451, Harlan Laboratories, Madison, WI), iii) standard diet with butylated hydroxyl anisole (BHA, 0.1% w/w) or oleanolic acid (0.1% w/w), iv) LD with butylated hydroxyl anisole (0.1% w/w) or oleanolic acid (OA, 0.1% w/w) for 6 weeks, BHA and oleanolic acid treatments starting first. At six weeks, livers and blood were collected.

Lipid Extraction From Liver Tissue: Cholesterol and triglycerides were extracted from liver. First, 150-200 mg of liver tissue was homogenized in a 15 mL polypropylene tube containing 3 mL of chilled PBS. 200 µL of the homogenate was transferred into a second 15 mL polypropylene tube preloaded with 3.75 mL of CHCl3 – MeOH (2:1). The tubes were vigorously mixed for 20 seconds, 0.5 mL of distilled water was added, and then mixed again for 15 seconds. The tubes were centrifuged for 5 minutes at room temperature and spun at 3000 rpm. The chloroform layer was transferred into a 5 mL glass tube and put into the speed vac1010 set at 45 °C for 2 hours to allow the liquid to evaporate. The lipid residue product was re-suspended in

200 µL of Ethanol containing 1% Triton X-100.

Cholesterol and Triglyceride Quantification: Cholesterol and Triglyceride quantifications were performed using colorimetric assay kits from Pointe Scientific Inc. according to the manufacturer's protocol. The lipid content was quantified indirectly by measuring the amount of formazan dye produced.

RNA isolation and quantitative real-time PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Camarillo, CA) according to the manufacturer's instructions. 1 µg of total RNA was converted to cDNA, and mRNA levels were quantified by quantitative real-time PCR using a Roche LightCycler 480 System (Roche Applied Science, Mannheim, Germany). SYBR green reagent was used, and relative target gene expression was normalized to GAPDH mRNA.

Western Blots. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to separate the proteins in each of the samples. The gel used was a 10% acrylamide resolving gel with a 4% acrylamide stacking gel. Polyvinylidene fluoride membranes were washed in Tris-buffered saline with Tween 20 (TBS/T), incubated with primary antibody specific to the protein of interest in 2% Non-fat dry milk in TBS/T at 4°C overnight. The membrane was washed again and then incubated with a secondary antibody specific to the primary antibody used in 2% Non-fat dry milk in TBS/T for 1 hour. The

membrane was then incubated with ECL+ chemiluminescent substrate (GE Healthcare Life Sciences, Pittsburgh, PA) and visualized using light sensitive film. The concentration of the proteins in each sample can be seen by quantification of the bands seen in the developed light sensitive film and normalized to β-actin.

Results

The effect of oleanolic acid on serum and hepatic cholesterol levels in mice. OA has been used in Chinese medicine for the treatment of liver disorders protecting against oxidative and electrophilic stress and activates the nuclear factor erythroid 2-related factor 2/ kelch-like ECH-associated protein 1 pathway inducing expression of cytoprotectant genes (Resiman, 2010).

The effect of oleanolic acid feeding on lithogenic diet-induced cholesterol uptake, synthesis, and transport gene expression in liver. Abcg5/abcg8 is a heterodimer responsible for cholesterol excretion from liver to the bile. Abca1 resides in the basolateral membrane transporting cholesterol back into the blood to lipid-poor lipoproteins. Ldlr is responsible for the uptake of cholesterol into the liver and hmg-coar is the rate limiting enzyme in which cholesterol is synthesized. The lithogenic diet alone increased abcg5/abcg8 mRNA expression 4-fold. OA administration with the lithogenic diet increased abcg5/abcg8 mRNA expression 3.5-fold. Abca1 mRNA expression was increased 2.5-fold on the lithogenic diet alone and 2-fold with OA on the lithogenic diet. Ldlr mRNA expression was slightly decreased on the lithogenic diet and more decreased with OA administration on the lithogenic diet. Hmg-coaR was decreased 3-fold and 5-fold on the lithogenic diet and lithogenic diet with OA respectively.

The effect of oleanolic acid feeding on lithogenic diet-induced bile acid uptake, synthesis, and transport gene expression in liver. Cyp7a1 is the rate-limiting enzyme in which cholesterol is converted to bile acids. Bsep is the bile salt export pumped from the liver into the bile. The lithogenic diet alone increased bsep mRNA expression slightly, and even more in the lithogenic diet with OA administration.

The effect of oleanolic acid feeding on Nrf2 and Nrf2-target gene expression in liver. OA administration significantly increased expression of nrf2 and its target genes in wild-type mice including nqo-1, gclc, and ho-1, however, not in nrf2-null mice (Reisman, 2010).

Conclusion

OA and BHA are two compounds that are considered to be Nrf2-activators. In In this study, adult male C57BL/6 mice were pair-wise fed *ad libitum* i) standard diet (CONT), ii) lithogenic diet (LD, 15% fat, 1.25% CH, 0.5% sodium cholate), iii) standard diet with oleanolic acid (0.1% w/w), iv) LD with oleanolic acid (OA, 0.1% w/w) for six weeks. BHA reduced body weight, however increased the liver weights of mice. BHA treatment doubled serum cholesterol levels in mice fed the lithogenic diet compared to mice that received lithogenic diet alone. Mice treated with OA in their diets have similar serum cholesterol levels to untreated mice. However, the overall finding of this study was that Oleanolic acid decreased hepatic cholesterol content.

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Xiaoling Yuan,1 Michael P. Shakarjian,4 Saeho Chong,2 and Ah-Ng Kong (2006) Induction of Heme Oxygenase-1 (HO-1) and NAD[P]H: Quinone Oxidoreductase 1 (NQO1) by a Phenolic Antioxidant, Butylated Hydroxyanisole (BHA) and Its Metabolite, tert-Butylhydroquinone (tBHQ) in Primary-Cultured Human and Rat Hepatocytes. Pharmaceutical Research 23: 2586-2594.

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Figures. Adult male C57BL/6 mice were pair-wise fed *ad libitum* i) standard diet (CONT), ii) lithogenic diet (LD, 15% fat, 1.25% CH, 0.5% sodium cholate), iii) standard diet with oleanolic acid (0.1% w/w), iv) LD with oleanolic acid (OA, 0.1% w/w) for six weeks. Mice were sacrificed and livers, gallbladders and serum were collected. The serum cholesterol levels were determined by colorimetric assay. The cholesterol contents were quantified by measuring the amount of formazan dye produced. Asterisks (*) represent a statistical difference ($p < 0.05$) between standard diet and lithogenic diet groups. Double Daggers (‡) represent a statistical difference ($p < 0.05$) between OA and the controls. The lithogenic increased serum cholesterol levels in controls (CONT) fed diet only by 40%. BHA treatment doubled serum cholesterol levels in mice fed the lithogenic diet compared to mice that received lithogenic diet alone. Mice treated with OA in their diets have similar serum cholesterol levels to untreated mice.

Effect of Nrf2 activators on body, liver, and gallbladder weight after feeding a lithogenic diet

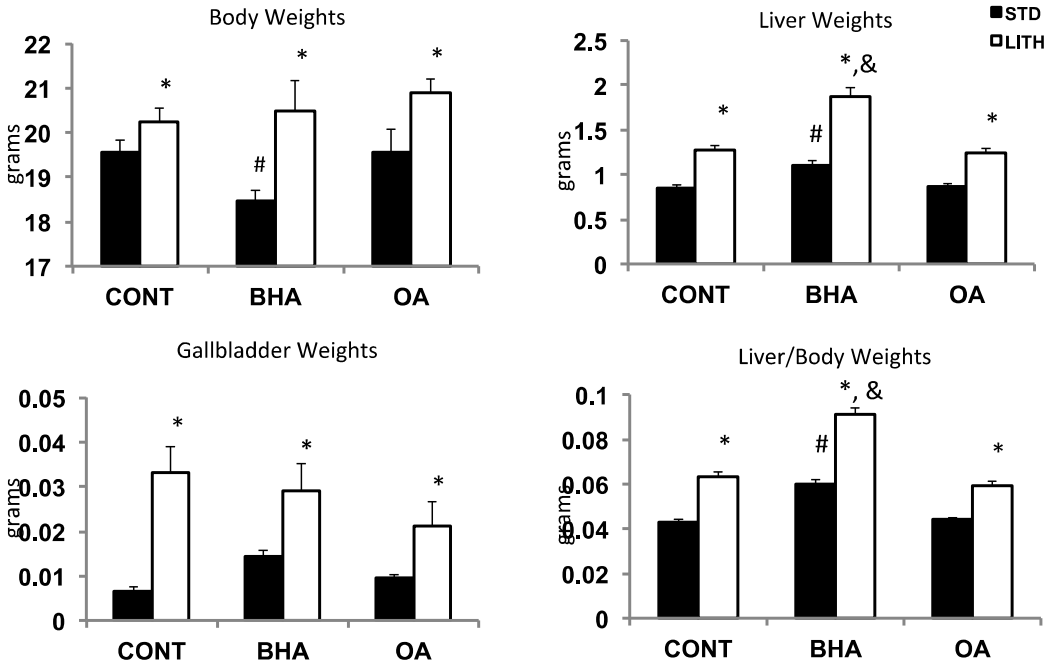


FIGURE 1

BHA and OA decreased hepatic cholesterol levels

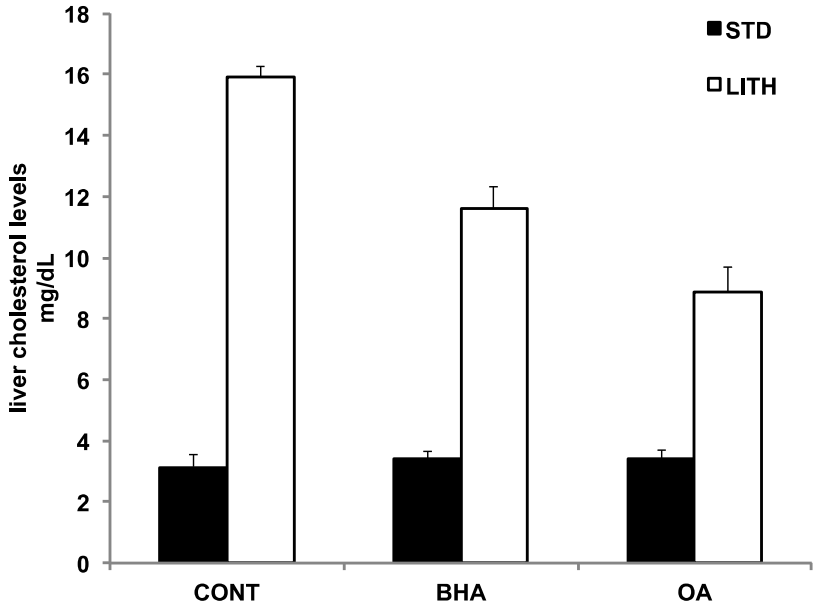
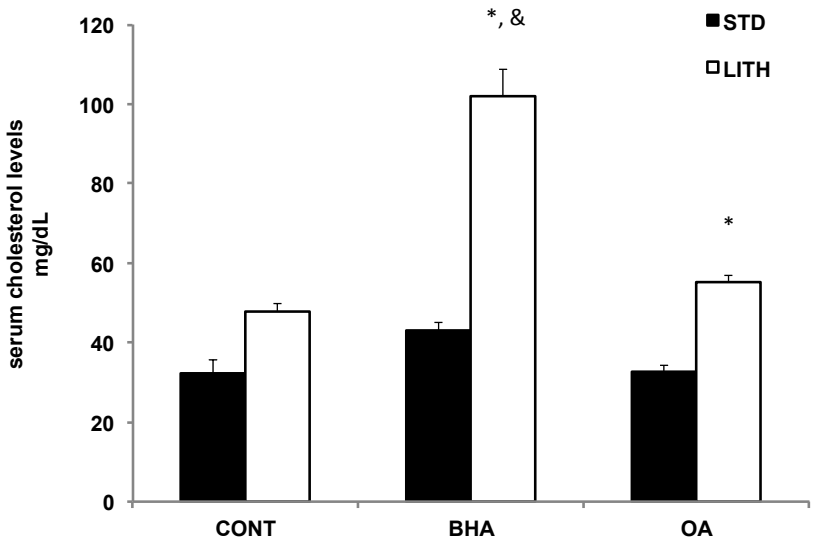
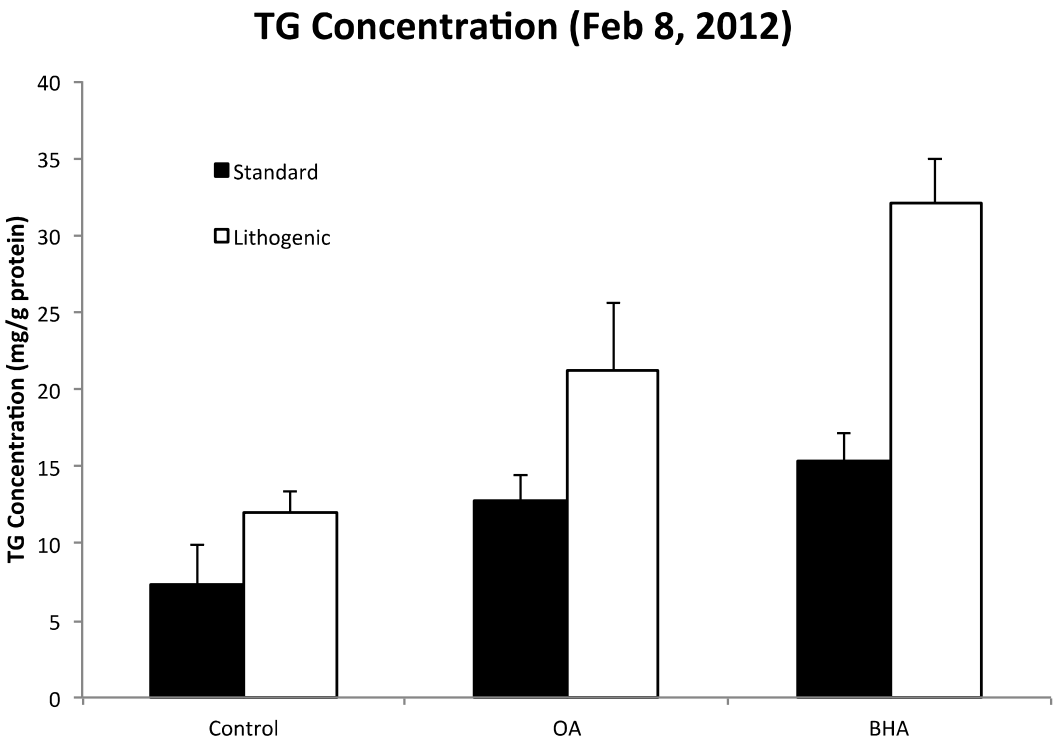
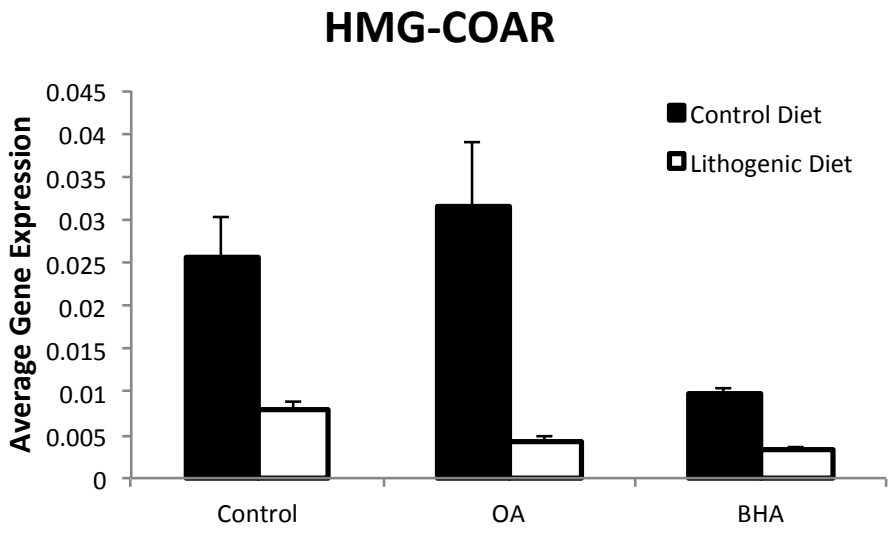


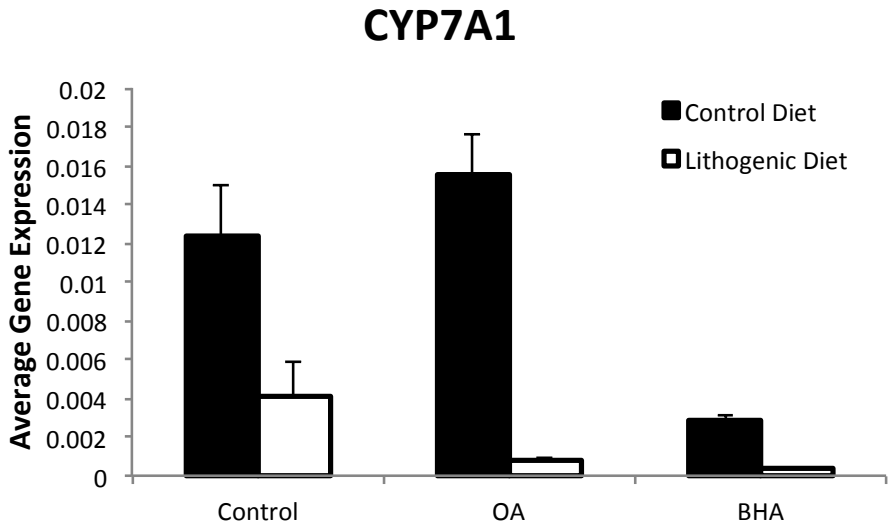
FIGURE 2

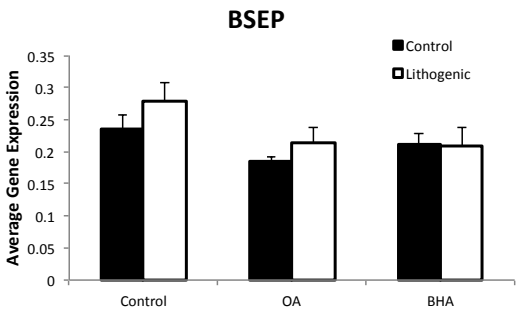
BHA and OA elevate serum cholesterol levels in C57Bl/6 mice

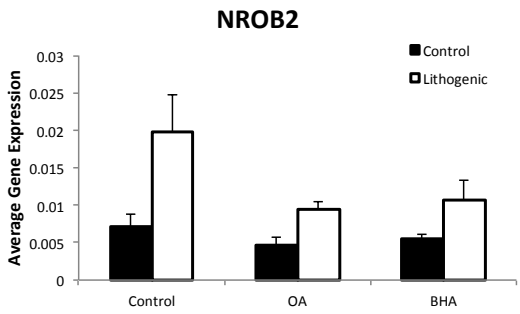


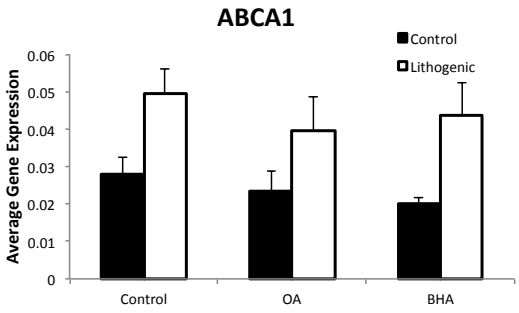

TG Concentration (Feb 8, 2012)
TG Concentration (mg/g protein)
40
35
30
25
20
15
10
5
0
Standard
Lithogenic
Control
OA
BHA

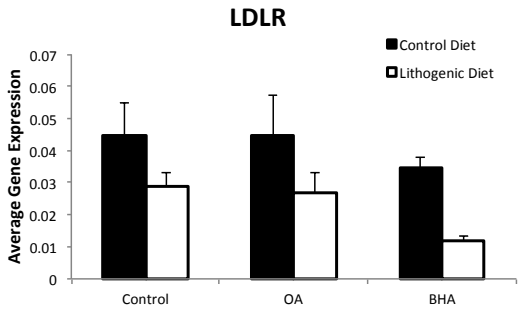

HMG-COAR
Average Gene Expression
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Control Diet
Lithogenic Diet
Control
OA
BHA


CYP7A1
Average Gene Expression
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Control Diet
Lithogenic Diet
Control
OA
BHA


BSEP
Control
Lithogenic
Average Gene Expression
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
OA
BHA


NROB2
Control
Lithogenic
Average Gene Expression
0.03
0.025
0.02
0.015
0.01
0.005
0
Control
OA
BHA


ABCA1
Control
Lithogenic
Average Gene Expression
0.06
0.05
0.04
0.03
0.02
0.01
0
Control
OA
BHA


LDLR
Control Diet
Lithogenic Diet
Average Gene Expression
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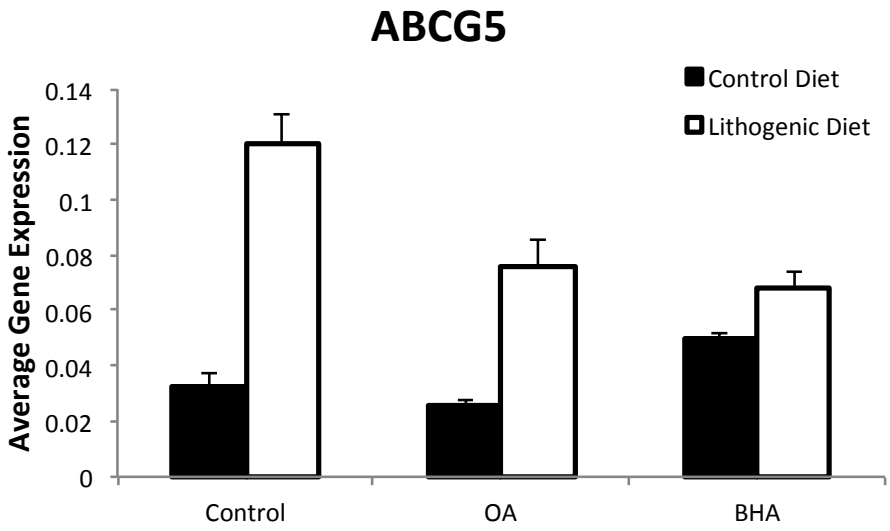
0.06
0.05
0.04
0.03
0.02
0.01
0
Control
OA
BHA


ABCG5
Control Diet
Lithogenic Diet
Average Gene Expression
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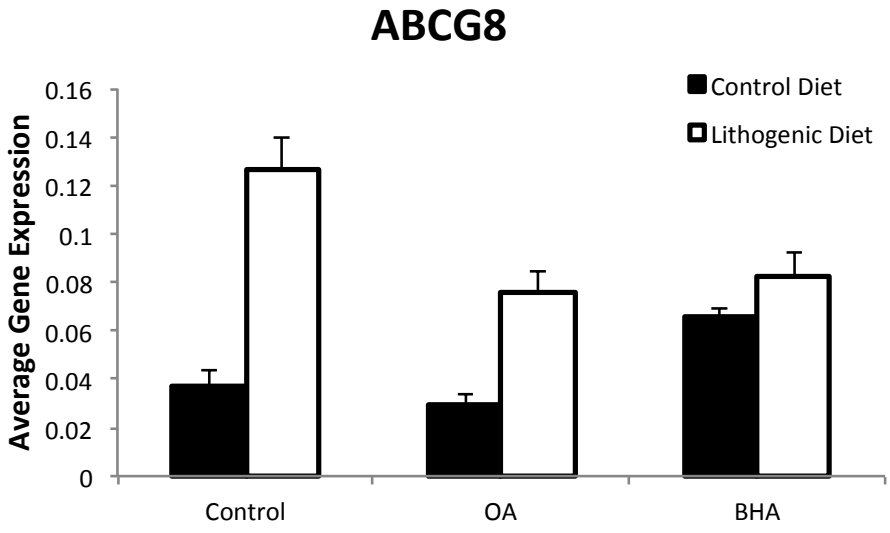
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
OA
BHA


ABCG8
Control Diet
Lithogenic Diet
Average Gene Expression
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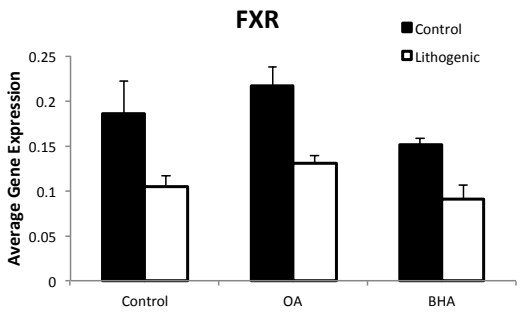
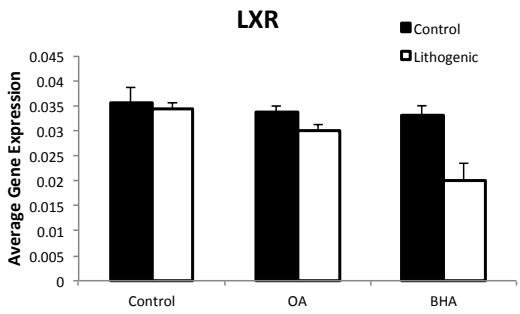
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
OA
BHA


FXR
Control
Lithogenic
Average Gene Expression
0.25
0.2
0.15
0.1
0.05
0
Control
OA
BHA


LXR
Control
Lithogenic
Average Gene Expression
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0.035
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0.025
0.02
0.015
0.01
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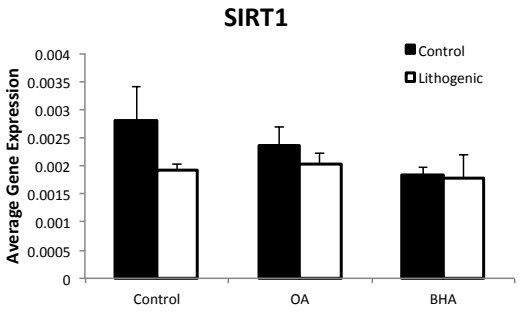
0
Control
OA
BHA


SIRT1
Control
Lithogenic
Average Gene Expression
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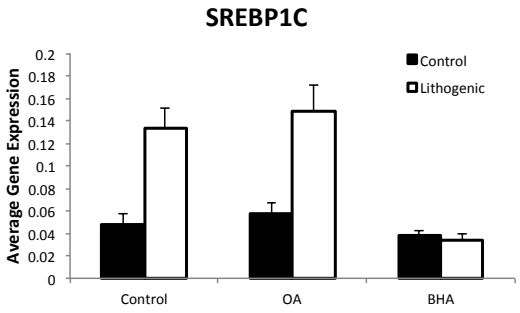
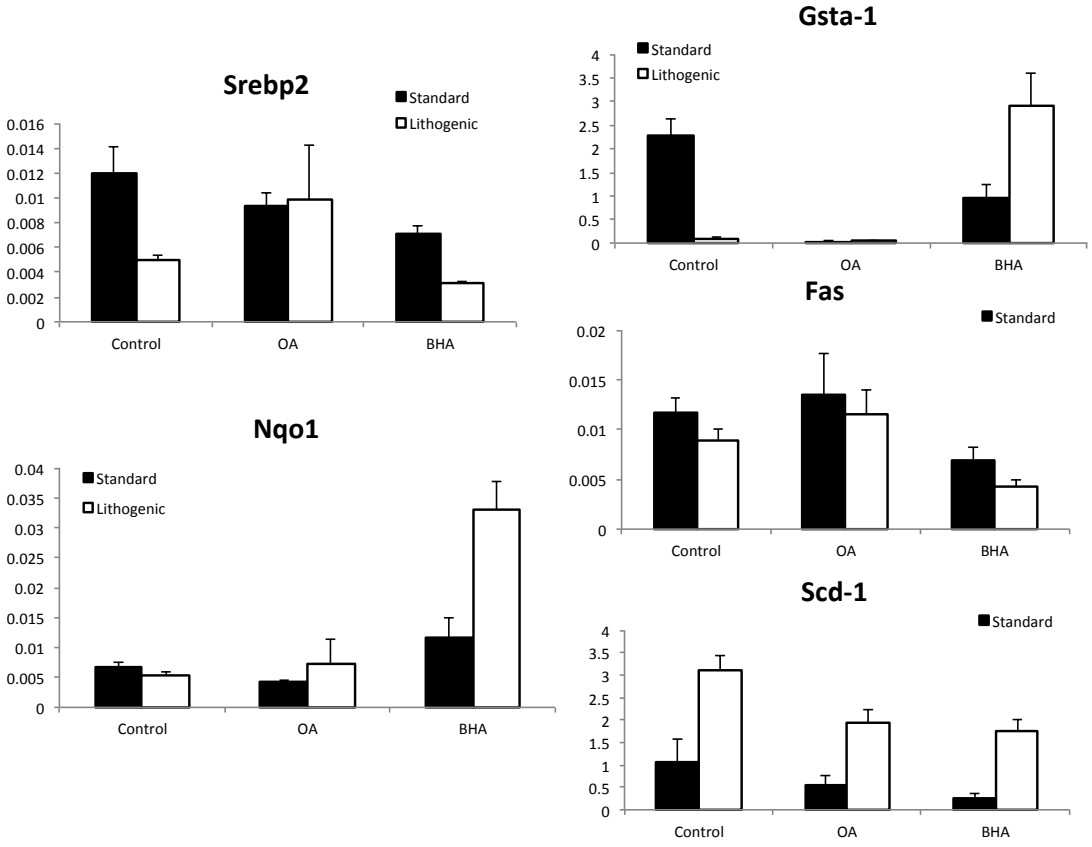
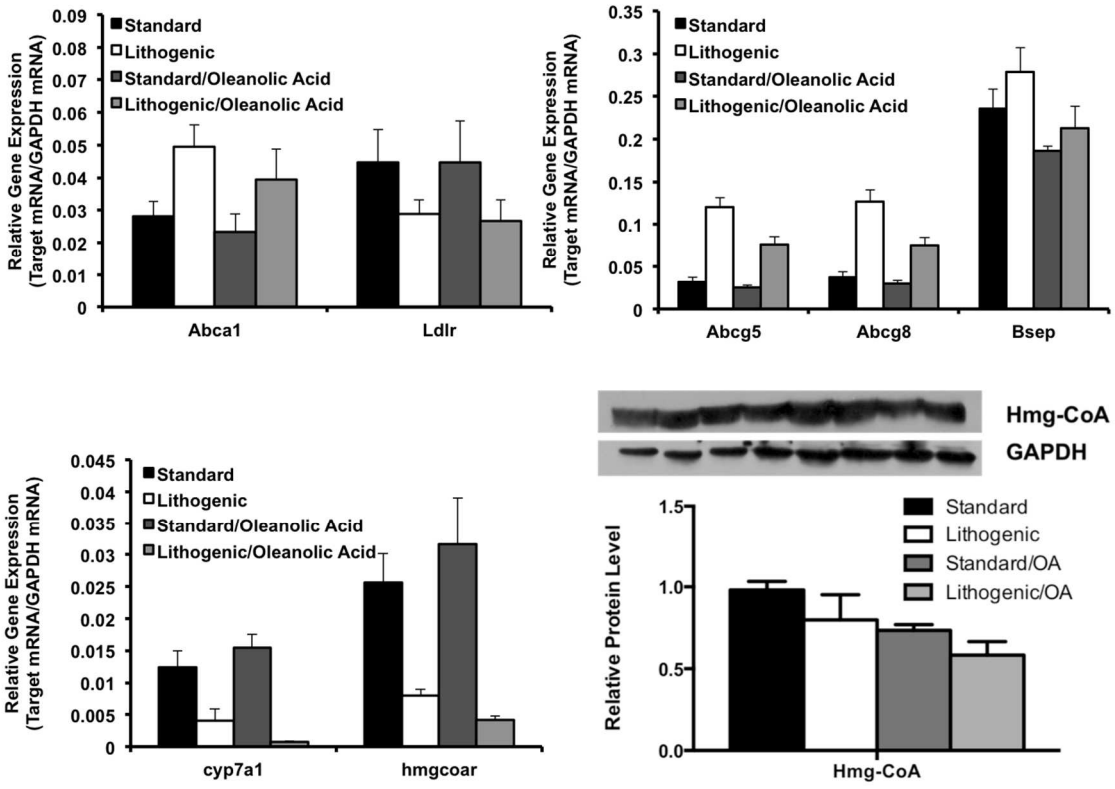
0
Control
OA
BHA


SREBP1C
Control
Lithogenic
Average Gene Expression
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
OA
BHA


Srebp2
Standard
Lithogenic
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Control
OA
BHA
Gsta-1
Standard
Lithogenic
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
OA
BHA
Nqo1
Standard
Lithogenic
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Control
OA
BHA
Fas
Standard
0.02
0.015
0.01
0.005
0
Control
OA
BHA
Scd-1
Standard
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
OA
BHA


Relative Gene Expression (Target mRNA/GAPDH mRNA)
Standard
Lithogenic
Standard/Oleanolic Acid
Lithogenic/Oleanolic Acid
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Abca1
Ldlr
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Abcg5
Abcg8
Bsep
Hmg-CoA
GAPDH
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
cyp7a1
hmgcoar
Relative Protein Level
Standard
Lithogenic
Standard/OA
Lithogenic/OA
1.5
1.0
0.5
0.0
Hmg-CoA