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

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

OF

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DEAN OF THE GRADUATE SCHOOL

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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
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.

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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.

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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.

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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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Chapter 1

Introduction to liver disease and perfluorinated compounds

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).

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,
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).

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 C1-3: Schematic depicting molecular mechanisms in caloric restriction and excess.](image-url)
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.
References: Chapter 1


combustion, hypolipidemia, inflammation and cancer. Nucl Recept Signal 8:e002.


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Chapter 2

MANUSCRIPT I

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

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Running title: PFOS interferes with caloric restriction

Manuscript in Preparation

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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 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.
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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.

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

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.
Body Weight

Percent Change (% BW Compared to Week 0)

Week

AL-Veh
CR-VEH
AL-PFOS
CR+PFOS

BW AUC

AUC

AL-VEH
CR-VEH
AL-PFOS
CR-PFOS

Liver Weight

Weight (grams)

AL-VEH
CR-VEH
AL-PFOS
CR-PFOS

WAT weight

Weight (grams)

AL-VEH
CR-VEH
AL-PFOS
CR-PFOS
A  Glucose in Media of 3T3L1 Cells

B  Adiponectin in Media of 3T3L1

C  Glucose in Media of Human Adipocytes

D  [Images of cell cultures]
Chapter 3

MANUSCRIPT II

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

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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

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**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 proliferator-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 acyltransferase (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-L1 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°C 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

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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.
References


23. Rosen, MB et al. (2008b) Toxicogenomic dissection of the perfluorooctanoic acid transcript profile in mouse liver: evidence for the
involvement of nuclear receptors PPAR alpha and CAR. Toxicol Sci. 103(1):46-56.


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.
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). CYP7A1 is 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-dehydroxylase or it can be converted to steroids by CYP11A1 (Tiangang, 2008). Before the cholesterol can be engulfed into the enterocytes, 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 sitosteroolemia, 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 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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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 2
BHA and OA elevate serum cholesterol levels in C57Bl/6 mice
HMG-COAR

Control Diet
Lithogenic Diet

Average Gene Expression

CYP7A1

Control Diet
Lithogenic Diet

Average Gene Expression
Srebp2

Nqo1

Gsta-1

Fas

Scd-1

Control
OA
BHA

Control
OA
BHA

Control
OA
BHA

Control
OA
BHA