Changes in Energy Metabolism Induced by PFOS and Dietary Oxylipins

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CHANGES IN ENERGY METABOLISM INDUCED BY PFOS AND DIETARY OXYLIPINS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

by
William Andrew Evans Jr.
May 2023

Accepted by:
Dr. William S. Baldwin, Committee Chair
Dr. Lisa Bain
Dr. Zhicheng Dou
Abstract

Perfluorooctanesulfonate (PFOS), an emerging environmental toxicant in addition to 9-hydroxyoctadecadienoic acid (9-HODE) and 9-hydroxyoctadecatrienoic acid (9-HOTrE), endogenously produced oxylipins from polyunsaturated fatty acids (PUFAs) by Cytochrome P450 2B6 (CYP2B6) have been recently associated with hepatic steatosis and peroxisome proliferator-activated receptor signaling by data produced from our laboratory and others. Given the importance of mitochondrial energy metabolism in the maintenance of lipid homeostasis, an array of extracellular flux assays was performed using HepG2 cells to test for the ability of these compounds to disrupt overall mitochondrial activity and respiration associated with fatty acid oxidation and glycolysis specifically. PFOS and 9-HOTrE increased spare respiratory capacity and various parameters of glycolysis including non-glycolytic acidification, while 9-HODE exhibited no effect on the mitochondria. 9-HOTrE also reduced HepG2 cells sensitivity to etomoxir, an inhibitor of CPT1A, indicating reduced basal fatty acid metabolism. All three compounds drastically increased triglyceride accumulation in HepG2 cells when supplemented with oleic acid, while the oxylipins additionally increased pyruvate concentrations, further associating these compounds and CYP2B6 with steatosis. 9-HODE perturbed gene expression of CD36, PPARγ, FASN and FOXA2, providing mechanistic insight into the triglyceride accumulation observed and further implying PPAR signaling, while a lipogenic SREBF-FASN or FASN coupled with a decrease in fatty acid oxidation pathway appeared to be more sensitive to PFOS and 9-HOTrE treatment, respectively. This study revealed that all three compounds can induce triglyceride accumulation in HepG2 cells while providing putative mechanistic data that their effects occur through different pathways within hepatocytes.
Figure A.1: Graphical abstract demonstrating the putative mechanisms of action of the exogenous toxicant PFOS plus the endogenously CYP2B6-produced oxylipins 9-HODE and 9-HOTrE.
Dedication

This thesis is dedicated to all my family and friends who have supported me not only in my education, but in every aspect of life. To all my friends in South Carolina, North Carolina and beyond, aunts and (sometimes crazy) uncles, my brother and his wife, and all others, thank you for your friendship and support through these past few years.

My dad has always been my hero. As a physician, he has been a key motivator in my pursuit of becoming a dentist, and as a father, he has set an example of the man I will always aspire to become. My mom’s sacrifice of her time to drive my brother and I to everything from school to sports and appointments, and to cook, clean, and pray for us is only partially representative of her unwavering love for us. I would never have been in this position to succeed at Clemson if it were not for their support and investment in me, and most importantly for teaching me to never give up on solving a problem or achieving a goal, no matter how difficult it may seem or how long it may take.

However, I most importantly would like to dedicate this thesis to my wife, Madison Evans. While she has always been supportive of my pursuit of dentistry, graduate school was entirely a detour for us. She has been my rock through the highs, the lows, and everything in-between. While I could describe her sacrifice of moving to a new state and finding a new job a year into our marriage, countless hours teaching and working on projects for her school, going for long periods of time “without” a husband as I worked on graduate school, studied for the DAT or applied to dental school, nothing I could write would truly convey the point. She is incredible. Madison, we did it.
Acknowledgements

I would like to acknowledge several people at Clemson who were vital and indispensable to this work. First, Dr. William Baldwin has been an incredible mentor and taught me not only the ins and outs of scientific procedures and techniques, but how to be a scientist. I have been able to learn from him the analyzing, the thinking, the progression important to developing hypotheses based on data and designing experiments to test those hypotheses and have truly grown to be a stronger scientist from my experience in this lab.

This work would also have not been made possible without Jazmine Eccles who took me under her wing, mentored and supported me, especially during the early days. Thank you for teaching me to perform qPCR and all the pre-requisite steps, gain footing in the Baldwin lab, and for the countless hours of discussing science, life, and the status quo of graduate school. Several other lab members have come and gone in the Baldwin lab, including Nathanial Westbrook and Manav Shah, who would often help me find things or work equipment when I first began, Lanie Williams, Bricen Ghent, and Ty Davis, whose time in the lab has overlapped the most with my own and have provided incredible amounts of support, as well as Ellie Anderson and Hannah Farrell, who have most recently joined the lab but fit in seamlessly and consistently enrich our lab’s culture.

I would like to thank Dr. Lisa Bain for being a member of my committee and all her lab members who have provided support and assistance with culturing cells along with allowing me to use their cell culture facility. I would also like to thank Dr. Zhicheng Dou for being a member of my committee and all his lab members who have assisted me and allowed me to work in their lab to use their Seahorse analyzer.
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Chapter 1: Background

PFOS History and Overview
Perfluorooctanesulfonate (PFOS), one of several thousand types of per- and poly-fluorinated alkylated substances (PFAS), has been formerly used in an array of industrial and consumer products and applications over the back half of the 20th century and beginning of the 21st that include fireproofing, waterproofing, stain repellents, and varnishes, among others (Leubker et al. 2005, Apelberg et al. 2007, Yu et al. 2009, Li et al. 2018, Gluge et al. 2020). Despite its ceased production in the United States since the early 2000s, PFOS is still manufactured in other countries including China and Brazil (Saikat et al. 2013, Gilljam et al. 2016, Liu et al. 2017).

As an emerging environmental toxicant, PFOS is associated with many adverse health effects in humans and rodents including hepatic steatosis accompanied by a decrease in white adipose tissue (WAT) and progression from steatosis to non-alcoholic steatohepatitis (NASH), a more severe, inflammatory state on the non-alcoholic fatty liver disease (NAFLD) spectrum (Pablos et al. 2015, Hamilton et al. 2021). Although the effects of PFOS on the liver and metabolic disfunction in general are often highlighted, other effects such as endocrine disruption, immunotoxicity, developmental and reproductive toxicity or perturbation of the gut microbiome are also notable, as PFOS has been demonstrated to disrupt estrogen and thyroid hormone signaling, act as an immunosuppressor, limit both pre- and post-natal growth and development of rats, impede cardiogenesis, and impair mucus formation leading to alterations in gut bacterial species ratios, to list a few (Apelberg et al. 2007, Cheng et al. 2013, Du et al. 2013, Dong et al. 2016, Gillois et al. 2018, DeWitt et al. 2019, Baldwin et al. 2020).
Persistence of PFOS and Routes of Exposure

The environmental persistence and bioaccumulation of PFOS and other PFAS are cause for their continued concern, particularly regarding effects of their chronic exposure (Saikat et al. 2013, Liu et al. 2017). This persistence is largely due to the several stable carbon fluorine bonds that are repeatedly found within the molecules’ structure (Gluge et al. 2020). PFOS concentrations of nearly 20 µg/kg dried weight in sediment and thousands of µg/L in water have been documented (Casado-Martinez et al. 2021, Wan et al. 2022). While these values represent extremes, a recent study estimates combined PFOS and perfluorooctanoic acid (PFOA) concentrations may exceed 10 ng/L in a large proportion and 1 ng/L in the majority of American tap water (Andrews and Naidenko 2020). For perspective, the health advisory level set by the Environmental Protection Agency (EPA) is a combined PFOS and PFOA concentration of 70 ng/L, and several states have established lower advisory levels (Andrews and Naidenko 2020).

In parallel with PFOS accumulation in water, high PFOS concentrations in fish have also been reported (Langberg et al. 2022). Additionally, use of aqueous film-forming firefighting foams (AFFF) represents a considerable source of PFAS contamination in water, with highest exposure areas including waters near airports or fire fighter training facilities (Mussabek et al. 2019, Langberg et al. 2022). For example, a PFOS concentration of 1,500 µg/kg of body weight in whole perch (Perca fluviatilis) was recorded near a Swedish airport (Langberg et al. 2022). More realistically, PFOS concentration in commercial fish may reach nearly 3 µg/kg (Augustsson et al. 2021). This is significant as fish consumption is commonly responsible for PFOS exposure in humans, although other common mechanisms of exposure do exist such as from drinking water,

**Distribution and Bioaccumulation of PFOS in Humans**

Following oral exposure, PFOS is readily absorbed into the blood where major sites of accumulation include blood, liver, and kidneys (Tahziz et al. 2021). The binding affinity of PFOS to serum proteins such as albumin, in addition to poor metabolism and excretion, is proposed as a significant cause of PFOS bioaccumulation in plasma, resulting in its detection in nearly 100% of Americans (Hamilton et al. 2021, Tahziz et al. 2021). These factors, coupled with elevated renal absorption, contribute to PFOS’ extensive half-life in humans of approximately 5.4 years (Xu et al. 2016). Furthermore, the liver is the main site of PFOS accumulation as liver PFOS concentrations are remarkably higher than kidneys or serum (Lau et al. 2007). This has been proposed to occur partially through extensive enterohepatic circulation following the excretion of PFOS into the biliary canaliculi (Wang et al. 2022).

**PFOS and NAFLD**

The effects of PFOS on the liver, particularly the disruption of triglyceride and cholesterol homeostasis have been a primary area of focus (Lau et al. 2007, Fragki et al. 2021). PFOS exposure in mice is associated with increased liver weight and triglyceride accumulation, peroxisome proliferation, and disruption of lipid metabolism (Lau et al. 2007, Fragki et al. 2021, Hamilton et al. 2021). These effects are hypothesized to occur through either disruption of fatty acid beta-oxidation, alterations of gene expression downstream of the peroxisome proliferator activated receptor alpha (PPARα), or a combination of both (Das et al. 2017, Fragki et al. 2021).
However, PPARα agonists such as the fibrates have been demonstrated to ameliorate certain properties of NAFLD in patients, and recent studies suggested that PPARα activation may be protective against PFAA-mediated steatosis (Nakagawa et al. 2012, Das et al. 2017, Choudhary et al. 2019, Fragki et al. 2021). Furthermore, transcriptional effects by PFOS observed in wildtype mice are still observed to a degree in PPARα-null mice, suggesting some perturbations in PPARα-independent pathways are controlling genes involved in maintenance of lipid homeostasis (Das et al. 2017). Finally, several issues must be taken into consideration when comparing the effects of PFOS in rodents to humans, such as the reduced sensitivity of PPARα to PFOS and PFOA in humans, reduced PPARα levels in human livers, and differences in length and types of exposures, diets, and kinetics of PFOS elimination (Gonzales et al. 1998, Fragki et al. 2021). Overall, PFOS’s mechanism of action in causing hepatic steatosis is unknown but may involve PPARα signaling, direct disruption of fatty acid utilization, or mitochondrial disruption (Wan et al. 2012, Fragki et al. 2021).

**Cytochrome P450 2B6**

Cytochrome P450 2B6 (CYP2B6) is an important detoxification enzyme in humans that metabolizes a vast array of xenobiotics from pharmaceuticals to pesticides and many endogenous substrates including bile acids, steroids, and polyunsaturated fatty acids (PUFAs) (Hodgson and Rose 2007, Wang and Tompkins 2008, Hamilton et al. 2021, Olack et al. 2022). Moreover, CYP2B6 displays a high degree of variability between individuals due largely to differences in regulation on both transcriptional and post-transcriptional levels and activity plus several known single nucleotide polymorphisms (SNPs) (Langmia et al. 2021). Each of these factors may
contribute to adverse drug reactions (ADRs) or variable effects of treatment with drugs that are CYP2B6 substrates (Desta et al. 2020, Langmia et al. 2021).

**CYP2B6 Role in Obesity, Fatty Liver, and PFOS Toxicity**

In addition to drug metabolism, CYP2B6 has recently been implicated in metabolic diseases such as obesity and NAFLD, although its importance or function in these cases is still poorly understood (Heintz et al. 2020, Krogstad et al. 2020, Heintz et al. 2022). CYP2B6 is regulated by multiple nuclear receptors and transcription factors including the constitutive androstane receptor (CAR), pregnane-X-receptor (PXR), glucocorticoid receptor (GR), and the forkhead box protein A2 (FoxA2), and disruption of several of these is associated with obesity (Hedrich et al. 2016, Heintz et al. 2019, Hamilton et al. 2021, Langmia et al. 2021). For example, CAR activation was demonstrated to combat many detrimental parameters of obesity and diabetes including hyperglycemia, insulin resistance, and hepatic steatosis (Dong et al. 2009). FoxA2 is positively regulated by fasting and fatty acids and negatively regulated by insulin and promotes transcription of genes involved in fatty acid oxidation (in addition to Cyp2b9 and CYP2B6), while suppressing gene expression of many lipogenic genes via inhibition of the mammalian “Target of Rapamycin” (mTOR) (Wolfrum et al. 2004, Bochkis et al. 2008, Hashita et al. 2008, Bochkis et al. 2013, Heintz et al. 2019).

Furthermore, Cyp2b9 is induced by a high fat diet (HFD), and CYP2B6 expression in the liver is inversely proportional to body mass index (BMI) (Hoek-van den Hil et al. 2015, Krogstad et al. 2020). Cyp2b-knockdown (Cyp2b-KD) mice have increased amounts white adipose tissue (WAT) compared to wildtype (WT) mice in addition to perturbations in the metabolism of unsaturated
fatty acids (UFAs) and other markers of lipid homeostasis including hyperlipidemia, elevated levels of cholesterol, and liver triglycerides with varying effects of sex and age (Damiri and Baldwin 2018, Krogstad et al. 2020, Heintz et al. 2022).

To further investigate the role of CYP2B6 in obesity and NAFLD, our laboratory has developed a Cyp2b-null mouse model which lacks the Cyp2b9, 10 and 13 genes, the murine hepatic CYP450s from the 2B family (Rosen et al. 2010, Dong et al. 2016, Kumar et al. 2017, Heintz et al. 2019). In a recent study, Cyp2b-null male mice exhibited increased body weight, WAT and liver triglycerides compared to their wild type (WT) counterparts under a HFD scenario (Heintz et al. 2019). No statistically significant differences between the two groups, other than liver triglycerides, were observed under a ND scenario (Heintz et al. 2019). However, ND-fed Cyp2b-null mice had similar lipid liver profiles of HFD-fed wildtype mice, indicating abnormal lipid metabolism in the Cyp2b-null mice. Cyp2b-null females showed no differences in body weight compared to the WT group by the final week of the study under either scenario (Heintz et al. 2019). Additionally, the role of CYP2B6 in the progression of NALFD may vary depending on sex with female Cyp2b-null mice showing greater resistance to hepatic steatosis compared to males (Heintz et al. 2020, Heintz et al. 2022).

Furthermore, our laboratory has developed a humanized CYP2B6-Transgenic mouse model (hCYP2B6-Tg), lacking Cyp2b9, 10 and 13 genes and containing human CYP2B6 (Hamilton et al. 2021). PFOS has been shown to activate CAR and PXR, both of which are major regulators of CYP2B6, and subsequently induce expression of Cyp2b9 and Cyp2b10 in mice (Rosen et al. 2010, Dong et al. 2016, Heintz et al. 2019). PFOS was also shown to induce CYP2B6 in the humanized
model (Hamilton et al. 2021). Differences in PFOS-mediated toxicity were observed between the models as CYP2B6 appeared to contribute towards increased PFOS retention in the serum and liver and the subsequent mortality of three hCYP2B6-Tg females during the study (Hamilton et al. 2021). PFOS increased serum alanine aminotransferase (ALT) and alkaline phosphatase (ALP), both of which were ameliorated by a HFD (Hamilton et al. 2021). Interestingly, CYP2B6 was protective against PFOS-mediated steatosis under a ND scenario, but exacerbated steatosis among PFOS/HFD co-treated mice (Hamilton et al. 2021). Under a HFD scenario alone, hCYP2B6-Tg mice were protected from weight gain over the course of the study, but hepatic triglycerides were increased in hCYP2B6-Tg male mice compared to Cyp2b-null mice indicating functional differences between murine Cyp2b6 and human CYP2B6 (Heintz et al. 2022).

**Functional Overview of Oxylipins**

Oxylipins are oxygenated metabolites of PUFAs that play important roles in biological signaling and many physiological functions such as inflammation, cellular differentiation and proliferation, vasoconstriction and dilation, and chemotaxis, with perhaps the most well-known oxylipins being the eicosanoids that consist partially of prostaglandins (PGs) and thromboxanes (TXs), both well-studied biomolecules (Gabb et al. 2015, Barquissau et al. 2017, Calder 2020). These actions occur primarily through autocrine and paracrine manners (Dennis and Norris 2015).

Due to their potency, oxylipins are synthesized *de novo* in a highly timed and regulated manner, rather than being utilized from storage (Nayeem 2018). Currently, there are three known oxylipin synthesis pathways, occurring via cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 (CYP) enzymes (Dennis and Norris 2015, Gabb et al. 2015). Regardless of the pathway,
oxylipin synthesis begins with release of its parent PUFA from the cell membrane via lipase (Gabbs et al. 2015, Barquissau et al. 2017). For example, arachidonic acid (AA, 20:4, ω-6) can be metabolized by each of these enzymatic pathways, with the COX pathway (commonly inhibited by non-steroidal anti-inflammatory drugs, NSAIDs) ultimately leading to production of PGE₂ and TXA₂, the LOX pathway ultimately leading to production of leukotrienes, and CYP/ω-hydroxylase or CYP/epoxyxygenase pathways ultimately leading to production of hydroxyeicosatetraenoic acids (n-HETEs) and epoxyeicosatrienoic acids (EETs), respectively (Bindu et al. 2020, Wang et al. 2021).

**CYP2B6 Oxylipins and Significance**

While oxylipins formed via COX and LOX pathways have been relatively well characterized over recent years, CYP-derived oxylipins have remained far less studied (Gilroy et al. 2016). However, the CYP2C and 2J families are generally considered to be strong producers of oxylipins, particularly from the metabolism of AA (Gilroy et al. 2016, Graves et al. 2019). Given the implications of CYP2B6 in metabolic disease, our laboratory recently studied PUFA metabolism by CYP2B6, which was shown to primarily metabolize the PUFAs linoleic acid (LA, 18:2, ω-6) and alpha-linolenic acid (ALA, 18:3, ω-3) to the oxylipins 9-hydroxyoctadecadienoic acid (9-HODE) and 9-hydroxyoctadecatrienoic acid (9-HOTrE), respectively, in addition to 13-position oxylipins (Figure 1.1) (Heintz 2020). While CYP2B6 preferentially metabolized ALA in baculosomes, metabolites of LA were far more abundant in the liver and serum of HFD-fed, hCYP2B6-Tg mice, indicating discrepancies in PUFA metabolism between in vitro and in vivo models (Heintz 2020). This may partially be due to differences in availability of each substrate from the animal’s diet as the high-fat diet contains nearly 20X more LA than ALA (Greupner et
al. 2018, Heintz 2020). These findings are uniquely relevant as both PUFAs are common components of dietary fat with LA being the most common in the human diet (Poudyal et al. 2013, Whelan and Fritsche 2013). While 9-HODE and 9-HOTrE potentially play contrasting roles in pain and inflammation, their effects on cellular energy metabolism have not been assessed (Nieman et al. 2014, Zhang et al. 2017). However, a positive relationship between oxylipins derived from ω-3 and ω-6 PUFAs via a CYP pathway and obesity has recently been uncovered, as well as between PUFAs derived from soybean oil and dysregulation of CYP450s and important fatty acid metabolism-associated genes such as Cyp3a, Cyp2c, and CD36 in mice (Deol et al. 2015, Deol et al. 2017).

![Figure 1.1](image)

**Figure 1.1:** Chemical structures of (A) 9-hydroxyoctadecadienoic acid (9-HODE) and (B) 9-hydroxyoctadecatrienoic acid (9-HOTrE).

**The Peroxisome Proliferator Activated Receptors (PPARs) and Their Ligands**

Oxylipins are one of several classes of ligands for the peroxisome proliferator activated receptors (PPARs), nuclear receptors that regulate gene expression associated with fatty acid uptake, metabolism, and synthesis in addition to cellular differentiation and proliferation (Kersten et al. 2000, Spector and Norris 2007, Muller et al. 2008, Buckner et al. 2021). Following activation by
ligand binding, PPAR heterodimers (with the retinoid-X-receptor, RXR) facilitate transcription with the help co-activators such as nuclear receptor co-activator 1 (SRC-1) or CBP/p300 at the peroxisome proliferator response element (PPRE) upstream of the target gene (Chandra et al. 2008, Viswakarma et al. 2010). Three isoforms, alpha (PPARα), beta/delta (PPARβ/δ) and gamma (PPARγ) exist and vary widely in the locations where they are expressed throughout the body (Kota et al. 2005).

**PPARα**

Although PPARα is expressed in many tissues such as skeletal muscle and adipose, it is primarily expressed in the liver and plays a crucial role in lipid oxidation and maintenance of blood triglyceride concentrations (Haluzik and Haluzik 2006, Todisco et al. 2022). This correlates with the function of many of its target genes that include the fatty acid-binding proteins (FABPs), peroxisomal acyl-coenzyme A oxidase 1 (ACOX), CYP4A10 and 14, and carnitine palmitoyltransferase I (CPT1A) (Rakhshandehroo et al. 2010, Dihingia et al. 2018). PPARα activity has been associated with increased insulin sensitivity and improvement of NAFLD, particularly in rodents (Haluzik and Haluzik 2006). Additionally, the role of PPARα in adaptation to fasting highlights its contribution to overall lipid homeostasis through oxidation and potentially the subsequent prevention of obesity, hepatic steatosis, and cardiovascular disease (Kersten et al. 1999, Berger et al. 2005, Fruchart 2005).

**PPARβ/δ**

PPARβ/δ is primarily expressed in skeletal muscle, although it is expressed in other tissues such as the skin, GI tract, and heart, as well as white and brown adipose tissue (Barquissau et al. 2017,
Manickam et al. 2020). Fatty acid binding proteins (FABPs) bind and carry ligands to PPARδ, aiding in its activation (Liu et al. 2018). PPARδ promotes the conversion of glycolytic to oxidative muscle fiber types coupled with an increase in mitochondrial biogenesis and upregulation of glucose transporter type 4 (GLUT4) during exercise (Montaigne et al. 2021). Furthermore, PPARδ activation increases overall fatty acid uptake and metabolism, providing resistance to obesity while maintaining important metabolic parameters overall such as insulin sensitivity (Liu et al. 2018). As such, the high metabolic demands of skeletal muscle, including glycolysis and fatty acid oxidation emphasizes the importance of PPARδ’s role in energy metabolism, as metabolic disruption in skeletal muscle can manifest systemically as in the case of obesity or diabetes (Manickam et al. 2021).

**PPARγ**

Like PPARα and β/δ, PPARγ is expressed in a variety of tissues including the liver, WAT and brown adipose tissue (BAT), colon, and spleen, although it is primarily expressed in adipocytes (Rogue et al. 2010, Janani and Kumari 2015). PPARγ regulates gene expression associated with glucose and fatty acid metabolism, inflammation, and adipogenesis (Marion-Letellier et al. 2016, Xie et al. 2020). Highlighting their role in fatty acid uptake, PPARγ ligands, such as thiazolidinediones (TZDs) improve insulin resistance by lowering the availability of free fatty acids as a fuel source through triglyceride uptake as a partial treatment for type 2 diabetes, albeit at the expense of weight gain and increased risk for cardiovascular disease (Abbas et al. 2012, Amato et al. 2012, Janani and Kumari 2015). Importantly, LA and its oxylipin derivatives are natural ligands for PPARγ (Marion-Letellier et al. 2016). Cluster of differentiation 36 (CD36), responsible for the uptake of fatty acids into the cell, is a notable target gene of PPARγ, and its
increased expression in the liver has been implicated in hepatic steatosis (Zhang et al. 2019). Additionally, increased PPARγ levels are observed in fatty liver while its inactivation ameliorates steatosis (Gavrilova et al. 2003). Although simplified, the combinatory uptake and oxidative functions of PPARγ and PPARα, respectively, has been a growing area of research due to its ability to improve both insulin sensitivity and fatty liver and provides an overview of each of their roles in the maintenance of lipid homeostasis (Xie et al. 2020).

**PFOS, Oxylipins, and the PPARs**

Given that a major hypothesis by which PFOS facilitates hepatic steatosis is via activation of PPARα, in addition to the known biological signaling roles of oxylipins and their actions on PPARs, our lab recently performed transactivation assays to test for the activation or inhibition of each specific PPAR by PFOS, 9-HODE and 9-HOTrE (Table 1.1) (Eccles 2022).

<table>
<thead>
<tr>
<th></th>
<th>PPARα</th>
<th>PPARβ/δ</th>
<th>PPARγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFOS</td>
<td>Potential Activator</td>
<td>Inhibitor</td>
<td>Weak Inhibitor</td>
</tr>
<tr>
<td>9-HODE</td>
<td>Strong Activator</td>
<td>No Effect</td>
<td>Inhibitor</td>
</tr>
<tr>
<td>9-HOTrE</td>
<td>Strong Activator</td>
<td>No Effect</td>
<td>Inhibitor</td>
</tr>
</tbody>
</table>

*Table 1.1. The activation or inhibition of the PPARs by PFOS, 9-HODE and 9-HOTrE.*
Current Research, Hypothesis, and Aims

Recent research from our laboratory demonstrated that CYP2B6 was protective against PFOS-mediated steatosis under a normal diet scenario but exacerbated steatosis under a high-fat-diet scenario. Additionally, CYP2B6 primarily metabolizes the PUFAs, LA and ALA, to the oxylipins 9-HODE and 9-HOTrE, respectively, with significantly greater measured serum concentrations of 9-HODE in humanized CYP2B6 mice. Our current data indicates that these oxylipins, in addition to PFOS act as activators or inhibitors of the PPARs, important nuclear receptors involved in the regulation of genes involved in fatty acid uptake, synthesis and metabolism. Given the association of these compounds with steatosis, we hypothesize that PFOS, 9-HODE and 9-HOTrE may contribute to hepatic steatosis by altering energy metabolism through activation or inhibition of the PPARs and subsequent changes in gene expression associated with fatty acid uptake, synthesis, and metabolism. The purpose of this study is to determine whether these compounds alter overall mitochondrial metabolism with specific analysis of both fatty acid beta-oxidation and glycolytic metabolic pathways, alter concentration of pathway intermediates, and test for changes in gene expression and activation of important signaling pathways for energy metabolism that may contribute to the steatotic phenotype observed during current and previous studies.

Aim 1: Test whether PFOS and select oxylipins alter overall mitochondrial activity.

Metabolic disruption is a hallmark of NAFLD. To test whether PFOS, 9-HODE and 9-HOTrE possess the ability to alter overall mitochondrial activity, Seahorse Mitostress Assays were performed in HepG2 cells using multiple doses of each compound. Additionally, a mitostress assay was performed using C2C12 cells treated with increasing doses of PFOS. The Seahorse XFe Analyzer measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)
that is primarily a result of electron transport chain progression (ETC) and the conversion of pyruvate to lactic acid, respectively. This assay provides an overall picture of metabolic activity within the cell. Additionally, palmitate oxidation assays were performed to test for changes in HepG2 cells’ ability to perform or dependence on fatty acid beta-oxidation for energy production following PFOS, 9-HODE, or 9-HOTrE treatment. This assay incorporates etomoxir, an inhibitor of Cpt1a that shuttles fatty acids into the mitochondria for metabolism. Similarly, glycolysis stress tests were performed to test for changes in glycolysis due to PFOS, 9-HODE or 9-HOTrE treatment. This test utilizes a substrate-limited medium and measures ECAR upon addition of glucose and inhibition of the ETC. Finally, triglyceride and pyruvate concentrations were measured to test for alterations by PFOS or the oxylipins that may contribute to or be a result of the metabolic changes observed during the Seahorse assays.

The Seahorse Mitostress Assay uses inhibitors to measure specific O₂ consumption in real-time.

- **Basal Respiration**: Oxygen consumption to meet metabolic needs under “normal” conditions.
- **ATP-Production Coupled Respiration**: Difference in rate between final basal measurement and first measurement after oligomycin injection.
- **Spare Respiratory Capacity**: Difference in rate between final basal measurement and first measurement after FCCP injection.
- **Maximal Respiration**: Difference in rates after FCCP and Rot./A.A. injections.

**Figure 1.2**: Outline and visual representation of the biological mechanisms used to measure mitochondrial metabolism by the Seahorse Mitostress Assay.
Aim 2: Determine putative mechanisms by which PFOS and select oxylipins alter energy homeostasis.

To test whether PFOS, 9-HODE or 9-HOTrE perturb gene expression with regards to fatty acid uptake, synthesis and metabolism, targeted qPCR was performed using RNA extracted from HepG2 cells following twenty-four-hour treatment of varying concentrations of these compounds. Genes targeted include PPARs and biomarkers of their induction (PPARα, ACOX, CPT1a, SCD-1, PPARγ, ANGPTL4, CD36), insulin signaling-associated genes (SREBF1c, FASN, FoxA2) and others relevant to fatty acid metabolism, cholesterol synthesis and glycolysis (HMGCR, HKII). These results may provide insight into the mechanisms contributing to the observed metabolic changes or alterations in triglyceride or pyruvate concentrations from the first aim. Using the gene expression data from the qPCR experiments, further experiments were conducted to determine potential mechanisms by which changes in gene expression and steatosis may be occurring. An ELISA utilizing an anti-pAMPK primary antibody was performed to test for changes in AMPK activation due to PFOS, 9-HODE or 9-HOTrE treatment at varying time points, as it is an important sensor of energy levels within the cells and promotes energy production through metabolism.
References


Chapter 2: PFOS Increases Triglyceride Accumulation and Expression of Lipogenic Genes in HepG2 Cells

Abstract

Perfluorooctanesulfonate (PFOS) is a prevalent compound found in fireproofing materials, seafood and tap water and has become increasingly associated with hepatic steatosis. Studies utilizing rodent models have suggested that activation of the peroxisome proliferator receptor alpha (PPARα) or potential inhibition of mitochondrial β-oxidation are the underlying mechanisms facilitating triglyceride accumulation in the liver. However, both the characteristics of PPARs and toxicokinetics of PFOS elimination drastically differ between rodents and humans. In the present study, Seahorse mitostress assays were performed to test for disruption of overall mitochondrial metabolism, glycolysis, and fatty acid β-oxidation. PFOS increased spare respiratory capacity (SRC) and multiple parameters of glycolysis in HepG2 cells while exhibiting no effect on palmitate metabolism while C2C12 cells were much more sensitive to PFOS overall. PFOS increased triglyceride concentrations in HepG2 cells in the presence of oleic acid-containing medium. qPCR was performed to test for changes in gene expression that may provide evidence for PPAR activation or inhibition in addition to perturbations in other metabolically important pathways such as the sterol regulatory element binding protein 1 (SREBF1) that may suggest alternative mechanisms facilitating steatosis in HepG2 cells by PFOS. No changes in gene expression regulated by PPARα was observed. However, expression of lipogenic and insulin signaling associated genes such as SREBF1, fatty acid synthase (FASN) and the forkhead box protein A2 (FoxA2) were upregulated in a similar fashion. Taken together, these data provide evidence for an alternative hypothesis that increased fatty acid synthesis may be the prominent contributing factor of PFOS-mediated steatosis in humans.
**Introduction**

Perfluorooctanesulfonate (PFOS) is an environmental toxicant that has been previously used in several industrial and consumer products and applications such as fireproofing, stain repellents, and varnishes (Li et al. 2018, Gluge et al. 2020). Production of PFOS was voluntarily ceased in the United States in 2002 due to its environmental persistence, bioaccumulation, and growing association with adverse health outcomes such as endocrine signaling disruption, developmental and reproductive toxicity, and hepatic steatosis (Apelberg et al. 2007, Gilljam et al. 2015, Chen et al. 2018, Baldwin et al. 2020, Hamilton et al. 2021). PFOS is still manufactured in other parts of the world, and its effects are still relevant due in part to its persistence in sediment and water (Saikat et al. 2013, Gilljam et al. 2016, Liu et al. 2017, Casado-Martinez et al. 2021, Wan et al. 2022). Drinking water and seafood consumption are major sources of PFOS exposure, and PFOS is detected in a vast proportion of American tap water and subsequently, serum (Fromme et al. 2009, Haug et al. 2011, Andrews and Naidenko 2020, Augustsson et al. 2021, Hamilton et al. 2021). The stability of PFOS’ chemical structure in addition to biological mechanisms such as its high binding affinity to serum albumin, high renal reabsorption, and extensive enterohepatic circulation contribute to the bioaccumulation of PFOS in serum and liver with a half-life in humans of approximately 6 years (Xu et al. 2016, Hamilton et al. 2021, Tahziz et al. 2021, Wang et al. 2022).

PFOS has been shown to induce hepatic steatosis in mice (Hamilton et al. 2021). Steatosis is defined as accumulation of fat in the liver exceeding 5% of its total mass and is the beginning stage of Non-Alcoholic Fatty Liver Disease (NAFLD), a disease that potentially affects up to 30% of Americans and can progress to increasingly detrimental states such as Non-Alcoholic
Steatohepatitis (NASH), cirrhosis or cancer (Fedchuck et al. 2014, Friedman et al. 2018). Although overconsumption and obesity are the main contributing factors to NAFLD, environmental factors are being increasingly determined to play roles as well (Rajak et al. 2022). To date, most research has studied PFOS-mediated NAFLD in rodent models, however, perfluoroalkyl substances (PFAS) have also been associated with steatosis in human cell lines (Pfohl et al. 2021).

The effects of PFOS on steatosis have been hypothesized to occur through two primary mechanisms: activation of the peroxisome proliferator-activated receptor alpha (PPARα) and/or the inhibition of fatty acid β-oxidation (Das et al. 2017, Fragki et al. 2021). The peroxisome proliferator-activated receptors (PPARs) are a logical target, as they regulate transcription of genes necessary for fatty acid uptake and metabolism, and their ligands include fatty acids, which the molecular structure of PFOS largely resembles (Marion-Letellier et al. 2016, Zhang et al. 2019, Gluge et al. 2020). PFOS has been shown to increase expression of genes that are regulated by PPARα in mice such as cytochrome p450 4A14 (CYP4A14), cluster of differentiation 36 (CD36) and peroxisomal acyl-coenzyme A oxidase 1 (ACOX) (Das et al. 2017, Hamilton et al. 2021). However, PPARα activation by pharmaceuticals has been demonstrated to be protective of NAFLD, and gene expression changes observed following PFOS treatment are still present in PPARα-null mice, indicating PFOS may exert effects independent of PPARα that contribute to steatosis (Das et al. 2017, Choudhary et al. 2019). The potential disruption of fatty acid β-oxidation by PFOS has been studied to a lesser degree, and its effects are less clear as some recent data suggests that PFOS may inhibit β-oxidation, while others suggest that PFOS and other PFAS have no or only a slightly negative effect (Wan et al. 2012, Cheng et al. 2016, Das et al. 2017).
Given these discrepancies and the growing concern of NAFLD worldwide, the purpose of this study is to better determine the energetic effects and molecular mechanisms by which PFOS facilitates steatosis utilizing the human cell line, HepG2 (Friedman et al. 2018). This is paramount as the effects of PFOS observed in rodent models may not be entirely applicable to humans given the lower expression of PPARα, reduced sensitivity to PFOS, and decreased excretion of PFOS by humans (Gonzales et al. 1998, Fragki et al. 2021). Our laboratory recently performed transactivation assays that demonstrated PFOS activates PPARα and inhibits PPARδ and PPARγ (Eccles 2022). All three isotypes are expressed in the liver, and PPARδ is uniquely significant as it is crucial for regulation of energy metabolism in skeletal muscle which contributes greatly to overall metabolic health (Manickam et al. 2021). Due to the evidence of PPARα activation by PFOS, we hypothesize that PFOS perturbs lipid homeostasis through the disruption of PPAR signaling and dysregulation of genes necessary for fatty acid uptake, synthesis, and metabolism, in addition to repressing overall mitochondrial metabolism.
Materials and Methods

Cell Culture
HepG2 cells (AddexBio, San Diego, CA) were cultured in Dulbecco’s Modified Eagle Medium, (DMEM, 4.5 g/L glucose and sodium pyruvate, Corning, Corning, NY) supplemented with 1% insulin-transferrin-selenium (ITS, Corning, Corning, NY), 1% penicillin-streptomycin (Gibco Cell Culture, Grand Island, NY), 1% L-glutamine (Gibco), and 10% fetal bovine serum (Cytiva USA, Marlborough, MA) in a humidified environment at 37°C with 5% CO₂ (Rungta et al. 2011). Cells were passaged using 0.25% trypsin (Gibco) upon reaching 70% confluency. C2C12 myoblasts (ATTC, Manassas, VA) were cultured as described above and passaged using 0.25% trypsin upon reaching 60% confluency (Stiffens et al. 2011).

Gene Expression Experiments and Quantitative Real-Time PCR (qPCR)
HepG2 cells were seeded in 6-well plates and grown to 1x10^6 cells per well before harvesting and extracting RNA. Twenty-four hours prior to RNA extraction, wells were treated with 0, 1 or 5 µM PFOS with DMSO as the carrier and provided in the control group (n = 6). Cells from each well were harvested as a sample using Accutase (Invitrogen, Carlsbad, CA USA), and total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Samples were then quantified and assessed for purity using a Nanodrop (Thermo Scientific, Waltham, MA). cDNA was synthesized with 2 µg of RNA from each sample by first diluting to a total volume of 25 µL using DEPC-treated water (Corning, Corning, NY) and incubating at 65°C for 5 minutes followed by addition of 25 µL reverse transcription enzyme solution consisting of 5 µL 5X RT Buffer, 1.625 µL dNTPs, 0.1 µL hexamers, 2 µL MMLV reverse transcriptase (Promega, Madison, WI), and 16.275µL DEPC water and incubation at 42°C for 60 minutes.
qPCR was performed by adding 1 μL of sample (diluted 1:10) to each well containing 12.5 μL SYBR Green Master Mix (Qiagen, Frederick, MD), 1μL forward primer, 1μL reverse primer, and 9.5μL of molecular grade H₂O. Plates were heated to 95.0°C for 30 seconds, followed by 50 cycles of 30 seconds denaturation (95.0°C), 30 seconds annealing (variable temperatures; all primer sequences and annealing temperatures are listed in Table 2.1), and 30 seconds elongation (72.0°C). A melt curve was performed for each plate to measure product purity, and a standard curve was performed using a mix of samples in 1:4 serial dilutions, 1:1-1:1024, to determine the efficiency of the reaction. Gene expression was normalized to the geometric mean expression of 18S and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by using the inverted Muller’s method (Muller et al. 2002, Roling et al. 2004).

| Table 2.1. qPCR primer information |
|-------------------------------|-------------------------------|-------------------------------|
| **Gene** | **Forward Sequence (5’ → 3’)** | **Reverse Sequence (5’ → 3’)** | **Annealing Temperature (°C)** |
| PPARα  | ACACCGAGGACTCTTGCAGA       | GGAAGGGCAAGTGCCGATG        | 61.6  |
| CD36   | TCTTTCTCGACGACCAATGT      | AGCCTCCTGTTCCAAGCAGTAGTGA  | 54.2  |
| ACOX   | GGCGCATACAAGAGGAGACCTG   | AGGTGAAAGCCCTTCCAGCCAGC   | 60.3  |
| SREBF-1c| GATGCGGAGAAGCTGCTATT      | GCTGTGTTGCAAGAAGCCGAA      | 61.0  |
| FASN   | TGCGTGGGCTTTGGAATGTG      | CTCCATGTCGTCGACGTCGAT      | 57.9  |
| PPARγ  | AAGCCCTTTCACTACTTGGA     | CAGGCTCCATTTGATTGG         | 55.0  |
| HMGR   | CTCTGTGAATGCTTTGATGG     | AGGCGAGGCAACAGCATGAT       | 55.2  |
| Cpt1a  | GATTTGCCTGTCGGCTTGG      | CTCTGTGCCTGAACTGTA         | 55.8  |
| ANGPTL4| GGACCACAAGACCTAGACCA     | GATCCCAAAAACCAGCAGTT      | 60.8  |
| SCD1   | TCTAGCTCTCATACACACCACCA  | TCGTCTCAACTTACTTCTCT      | 59.6  |
| FoxA2  | GGAAGCAGCTACTGACGACG     | CGGTCTCATGCGGTTCATCC       | 60.6  |
| HKII   | TGCCACAGAATTAACAGACG     | CCCGTGCCCAATGAGAC          | 58.8  |
| 18S    | ATGGCCGTCTTATATGGTGT     | ATGCCAGATGCTCGTCTGTT      | 64.0  |
| GAPDH  | CCTCTATTGAGCCTCACTA       | CTGGAAGATGCGTGATG          | 50.0  |

**Seahorse Assays**

Forty-eight hours prior to metabolic analysis, HepG2 cells were seeded in a Seahorse XFe24 well plate (Agilent Technologies, Santa Clara, CA) with growth medium as described above at a density of 7.5x10⁴ cells per well. Twenty-four hours prior to the mitostress assay, wells were treated with
0.2, 1 or 5 μM PFOS with a DMSO group included as the control (n = 5). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse XFe24 Analyzer (Agilent Technologies, Santa Clara, CA).

For Mitostress assays, just prior to the assay, cells were washed twice with assay medium consisting of Seahorse XF DMEM supplemented with 1 mM pyruvate, 2 mM glutamine and 10 mM glucose (Agilent Technologies, Santa Clara, CA), then incubated in a humidified, non-CO₂ environment at 37°C for 1 hour. Three initial OCR and ECAR measurements were taken following the injection of each inhibitor (oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and rotenone/antimycin a), and three measurements were taken prior to any injection to measure basal respiration. Basal respiration is defined as the difference between the last measurement prior to oligomycin injection and first measurement following rotenone/antimycin a injection. ATP-production coupled respiration is calculated as the difference between the last measurement prior to oligomycin injection and the first measurement following. Maximal respiration is defined as the maximum OCR following FCCP injection, and Spare Respiratory Capacity is defined as the difference between maximal and basal respiration.

C2C12 cells were seeded at a density of 2.5x10⁴ cells per well in a Seahorse XFe24 well plate (Agilent Technologies, Santa Clara, CA) with growth medium containing 2 or 6 μM PFOS with a DMSO group included as the control (n = 3 or 4). Cells were washed with and incubated in assay medium and OCR and ECAR were measured as described above.
For palmitate oxidation assays, twenty-four hours prior to the assay, growth medium was replaced with DMEM (without glucose and sodium pyruvate, Gibco, Grand Island, NY) supplemented with 10% FBS, 1% Pen-Strep, 1% L-glut, 300 nM sodium palmitate, 0.5 mM L-carnitine, 0.24 mM fatty acid free bovine serum albumin (BSA), and 2.25 nM sodium chloride containing 5 µM PFOS with a DMSO group included as the control (n = 5). Prior to the assay, cells were washed twice with assay medium consisting of Seahorse XF DMEM supplemented with 300 nM sodium palmitate, 0.5 mM L-carnitine, and 2 mM glucose (Agilent Technologies, Santa Clara, CA), then incubated in a humidified, non-CO₂ environment at 37°C for 1 hour. Cells were washed again with assay medium immediately prior to the assay. Three initial OCR and ECAR measurements were taken followed by injection of etomoxir into half of the assay wells which created four treatment groups as follows: treatment + assay media, treatment + etomoxir, control + assay media, and control + etomoxir. Six rate measurements were taken following etomoxir injection. Oligomycin, FCCP, and rotenone/antimycin a were then injected into the assay followed by three rate measurements each.

For glycolysis stress tests, twenty-four hours prior to the assay, cells were treated with 0.2, 1 or 5 µM PFOS with a DMSO group included as the control (n = 5). Prior to the assay, growth medium was changed for a substrate-limited assay medium containing Seahorse XF Base Medium (Agilent Technologies, Santa Clara, CA) supplemented with 2 mM glutamine, and cells were incubated in a humidified, non-CO₂ environment at 37°C for 1 hour. Measurements were performed as described earlier.
Triglyceride and Pyruvate Quantification

HepG2 cells were seeded in cell culture dishes at a density of $2.0 \times 10^6$ cells per dish and grown to 100% confluency. Forty-eight hours prior to reaching full confluency, cells were treated with 1 or 5 µM PFOS with a DMSO group included as the control ($n = 6$). For triglyceride quantification, cell culture medium was additionally supplemented with 300 nM oleic acid forty-eight hours prior to quantification. Triglycerides and pyruvate were extracted and quantified using colorimetric (triglycerides) and fluorometric (pyruvate) assays (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

pAMPK ELISA

HepG2 cells were seeded in a 96-well plate at a density of $3.0 \times 10^4$ cells per well and allowed to adhere overnight. Cells were treated with 5 µM PFOS 60 minutes prior to fixation with 4% formaldehyde, with a DMSO group included as the control ($n = 4$ or 5) Quantification of phosphorylated AMPK was performed using a cell-based, fluorometric ELISA (LS Bio, Seattle, WA) according to kit protocol and normalized to the total protein within each well.

Statistical Analysis and Preparation of Visuals

All statistical analysis and graph preparation was performed with GraphPad Prism 7.0 (San Diego, CA). Student’s t-tests were performed when comparing two groups and one-way ANOVA followed by Fisher’s LSD as the post-hoc test was performed when comparing more than two groups. Chemical structures were rendered using ChemDraw 21.0.0 (PerkinElmer, Shelton, CT).
Results

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) in HepG2 cells following PFOS exposure:

To test whether PFOS perturbs hepatic mitochondrial metabolism in the liver, Seahorse mitostress assays were performed with HepG2 cells. This assay measures the cellular oxygen consumption rate (OCR) that is a direct result of electron transport chain (ETC) activity during respiration (Mookerjee et al. 2015). PFOS increased spare respiratory capacity (SRC) in a dose-dependent manner, with no apparent effects on other parameters of mitochondrial metabolism (Figure 2.1). Calculated as difference between the maximal and basal respiration, SRC is a marker of cell health and adaptability and is influenced by several factors that include improved mitochondrial health, greater mitochondrial number or increased upstream energy sources such as triglycerides or pyruvate that fuel mitochondrial respiration through the formation of the intermediate, Acetyl-CoA (Marchetti et al. 2020).

Figure 2.1: PFOS increased Spare Respiratory Capacity in a dose-dependent manner. Data from Seahorse mitostress test in HepG2 cells treated with 0, 0.2, 1 or 5 µM PFOS 24 hours prior to the assay. Data were analyzed via one-way ANOVA followed by Fisher’s LSD and are expressed as mean ± SEM. *p<0.05. (n = 5).
Palmitate oxidation assays were performed to test the hypothesis that PFOS interferes with fatty acid metabolism and further characterize the metabolic phenotype of HepG2 cells following PFOS treatment. No significant differences were observed on any parameter of palmitate metabolism as both DMSO- and PFOS-treated cells performed similarly throughout the assay, showing decreased overall OCR following injection of the Cpt1a inhibitor, etomoxir, and vastly diminished maximal respiration following FCCP injection (Figure 2.2). These findings suggest that PFOS does not negatively interfere with mitochondrial β-oxidation in HepG2 cells.

Figure 2.2: PFOS exhibited no effect on palmitate oxidation. Data from Seahorse substrate oxidation assay in HepG2 cells treated with 0 or 5 µM PFOS 24 hours prior to the assay. Data were analyzed via one-way ANOVA followed by Fisher’s LSD and is expressed as mean ± SEM. ****p<0.0001. (n = 5).

As previously mentioned, Acetyl-CoA produced from glycolysis can also fuel the mitochondria, effecting the total OCR. A glycolysis stress test was performed to test for potential changes in glycolytic activity that may contribute to the changes in OCR observed during the mitostress test. Pyruvate produced from glycolysis is converted to lactic acid which results in acidification of the
cell culture medium and is measured as the extracellular acidification rate (ECAR) (Shah-Simpson et al. 2016). Additionally, acidification can occur due to the formation of carbonic acid (H$_2$CO$_3$) from carbon dioxide (CO$_2$) production as a byproduct of Krebs cycle progression, which also contributes to the OCR (Mookerjee et al. 2015). No changes in ECAR were observed following the injection of glucose (glycolysis) or oligomycin (glycolytic capacity), however, a significant increase in glycolytic reserve (the difference between glycolysis and the glycolytic capacity) occurred following the lowest dose (Figure 2.3). Furthermore, PFOS increased non-glycolytic acidification in a reverse dose-dependent manner (Figure 2.3). Non-glycolytic acidification is measured in the absence of glucose but presence of glutamine in the cell culture medium, indicating low concentrations of PFOS may be stimulating energy production from the metabolism of glutamine (Pavlou et al. 2017).

**Figure 2.3:** PFOS increased glycolytic reserve and non-glycolytic acidification in a reverse dose-dependent manner. Data from Seahorse glycolysis stress test in HepG2 cells treated with 0, 0.2, 1 or 5 µM PFOS 24 hours prior to the assay. Data were analyzed via one-way ANOVA followed by Fisher’s LSD and are expressed as mean ± SEM. *p<0.05, ***p<0.001. (n = 5).
Macromolecules and intermediates involved in mitochondrial metabolism:

Total triglycerides and pyruvate were isolated and quantified from HepG2 cells treated with increasing doses of PFOS for 48 hours to test for changes in important intermediates that may contribute to the metabolic changes observed with or without oleic acid present. The presence of oleic acid in the medium is a common technique for investigating potential NAFLD development in vitro (Xie et al. 2016, Guo et al. 2020). PFOS significantly increased triglyceride concentrations at both doses in the presence of oleic acid, while no significant increase in pyruvate was observed (Figure 2.4).

![Figure 2.4](image_url)

Figure 2.4: PFOS increased concentrations in a dose-dependent manner in the presence of oleic acid. Measured (A&B) triglyceride and (C) pyruvate concentrations from HepG2 cells treated with 0, 1 or 5 μM PFOS 48 hours prior to extraction or PFOS plus supplementation with oleic acid (B). Data are presented as mean ± SEM. Data were analyzed via one-way ANOVA followed by Fisher’s LSD and are expressed as mean ± SEM; **p<0.01, ***p<0.001 (n = 6).

Quantitative real-time PCR (qPCR):

To determine putative mechanisms by which PFOS alters mitochondrial metabolism, qPCR was performed to test for changes in gene expression of key energy utilization genes often regulated by the PPARs and SREBF1. PPARα regulates many genes that code for proteins involved in fatty acid uptake and oxidation such as ACOX, carnitine palmitoyltransferase 1A (Cpt1a), CD36, and
angiopoetin-like 4 protein (ANGPTL4) (Yoshida 2007, Rakhshanderoo et al. 2010, Dihingia et al. 2018, Wu et al. 2018). Although, ANGPTL4 and CD36 are regulated by other PPARs and transcription factors demonstrating at lease some potential convergence of pathways (Yoshida 2007, Zhou et al. 2008). No significant difference was observed in expression of PPARα, specific biomarkers of PPARα, or general PPAR biomarkers following 24-hour PFOS treatment in HepG2 cells (Figure 2.5).

SREBF1 is an important transcription factor that regulates genes involved in lipogenesis, often in response to feeding or insulin signaling, among others (Gosmain et al. 2005, Dif et al. 2006). These genes include fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1), and HMG-CoA-reductase (HMGCR) (Gosmain et al. 2005, Burg and Espenshade 2011, Mauvoisin and Mounier 2011, Figure 2.5: PFOS perturbed expression of lipogenic genes. Heatmap showing differentially expressed genes following increasing doses of PFOS compared to DMSO control (n=6). Data were normalized to the geometric mean of 18S and GAPDH and is expressed as log_2 fold change relative to control. Data were analyzed via one-way ANOVA followed by Fisher’s LSD. *p < 0.05.)
Additionally, SREBF1 positively regulates hexokinase II (HKII) and is an important part of the insulin signaling cascade along with the forkhead box protein A2 (FoxA2), although their function and regulation by insulin drastically differs (Gosmain et al. 2005, Kohjima et al. 2005). Finally, PPARγ regulates FASN and is involved in adipogenesis (Kawai et al. 2010, Ahmed et al. 2016). PFOS decreased expression of SCD1 in HepG2 cells at the highest concentration (Figure 2.6), and increased expression of SREBF1c, FASN, and FoxA2 at the lowest concentration. No changes were observed in PPARγ, HMGCR or HKII (Figure 2.5).

AMP-kinase phosphorylation following PFOS exposure:

AMP-activated kinase (AMPK) is a primary energy sensor and regulator within the cell and is activated by increased adenosine monophosphate (AMP) to adenosine triphosphate (ATP) (AMP:ATP) ratio, among other mechanisms (Hardie et al. 2012, Jeon 2016, Herzig and Shaw 2018). To test whether PFOS disrupts this sensitive and crucial pathway in HepG2 cells, a cell-based ELISA was performed to detect the presence of phosphorylated AMPK (p-AMPK), indicating activation (Herzig and Shaw 2018). PFOS induced a slight but significant increase in AMPK phosphorylation (Figure 2.6). The SREBF1c and FASN genes code for proteins that facilitate lipogenesis, an energetically demanding process. AMPK activation indicates a low energy state (low ATP) within the cell and upregulates genes necessary for energy production pathways such as phosphofructokinase 1 (PFK1), adipose triglyceride lipase (ATGL), and peroxisome proliferator-activated receptor gamma cofactor 1-alpha (PCG1α) while inhibiting lipogenic genes such as HMGCR, FASN and SREBF1 (Viollet et al. 2009, Hardie et al. 2012, Herzig and Shaw 2018, Liu et al. 2021). Thus, it was unexpected to observe an increase in p-AMPK following PFOS treatment based on the previous gene expression data.
Increased sensitivity of skeletal muscle OCR to PFOS:

PPARδ is highly expressed in skeletal muscle. Given our preliminary data indicating PFOS is a PPARδ inhibitor and skeletal muscle’s role in whole-body metabolism, a Seahorse mitostress assay was performed using C2C12 cells treated with increasing concentrations of PFOS. In a clear contrast with the effects observed in HepG2 cells, PFOS decreased basal respiration, ATP-coupled respiration and proton leak while increasing SRC in a dose dependent manner (Figure 2.7).

Figure 2.6: PFOS induced AMPK phosphorylation. Graph showing fluorescence from cell-based ELISA using anti-pAMPK primary antibody performed 60 minutes following incubation with 5 µM PFOS or 0.25 uL DMSO. Data were analyzed via t-test and are expressed as mean ± SEM. *p<0.05. (n = 5).

Figure 2.7: PFOS decreased Basal Respiration, ATP-Production Coupled Respiration and Proton Leak and increased Spare Respiratory Capacity in a dose-dependent manner in C2C12 cells. Data from Seahorse mitostress test in C2C12 cells treated with 0, 0.2, 1 or 5 µM PFOS 24 hours prior to the assay. Data were analyzed via one-way ANOVA followed by Fisher’s LSD and are expressed as mean ± SEM. *p<0.05. **p<0.01 (n = 5).
Only SRC was increased in HepG2 cells (Figure 2.1). This data potentially corresponds with our previous transactivation data (Eccles, unpublished). PPARδ activation promotes fatty acid uptake and catabolism in skeletal muscle and ameliorates metabolic diseases such as obesity and insulin resistance (Luquet et al. 2005). PFOS decreased overall basal and ATP-production coupled respiration, but likely does not inhibit overall metabolism or metabolic flexibility as an increase in SRC was still observed and maximal respiration trended upwards (Figure 2.7). However, this data still suggests PFOS negatively alters mitochondrial function in skeletal muscle which may potentially have systemic effects throughout the body.
**Discussion**

To our knowledge, this is the first study to analyze changes in OCR induced by PFOS in association with both glycolysis and fatty acid β-oxidation. PFOS perturbed overall mitochondrial metabolism in HepG2 cells with the strongest effects observed on glycolytic parameters (Figures 2.1, 2.2 and 2.3). Both SRC and glycolytic reserve were increased by PFOS, indicating metabolic flexibility (Figures 2.1 and 2.3) (Marchetti et al. 2020). An increase in non-glycolytic acidification was observed following treatment with the lowest concentrations of PFOS, which potentially indicates increased glutamine metabolism, as it is the only energy source available in the assay medium (Figure 2.3).

The liver, in addition to skeletal muscle, is a primary producer of glutamine (Cruzat 2019). Alpha-ketoglutarate is produced from glutamine through the intermediate, glutamate via glutaminases, glutamate dehydrogenase, and aminotransferases and can feed into the citric acid cycle for NADPH and subsequently energy production, gluconeogenesis from oxaloacetate, or lipid synthesis via citrate formation following reductive carboxylation. (Altman et al. 2016, Shah and Wondisford 2020). Carbon dioxide produced from the Krebs cycle acidifies the medium through formation of carbonic acid (Mookerjee et al. 2015).

Importantly, PFOS had no effect on palmitate metabolism in HepG2 cells (Figure 2.2). Other studies have proposed and provided data for the hypothesis that PFOS may negatively interfere with β-oxidation, as Wan et al. demonstrated that PFOS decreased β-oxidation despite upregulation of many PPARα controlled genes vital for fatty acid metabolism *in vivo*, and our laboratory further observed a downregulation of Cpt1a following PFOS exposure *in vivo* (Wan et
Downregulation of Cpt2 and the fatty acid binding proteins (FABPs) following PFOS exposure in chicken eggs in addition to a downregulation of acyl-CoA thioesterase 8 (ACOT8), which negatively regulates peroxisomal beta oxidation has also been observed (Jacobsen et al. 2018). This agrees with additional in vivo data suggesting PFOS may promote a switch between mitochondrial and peroxisomal β-oxidation (Jacobsen et al. 2018). However, isolated rat mitochondria experienced no significant effect with regards to mitochondrial metabolism following treatment with a panel of PFAAs (Das et al. 2017).

A large variation in model types and PFOS concentrations has been offered as an explanation for numerous amounts of contradicting data, and some studies have used PFOS concentrations and doses well above realistic levels (Jacobsen et al. 2018). PFOS concentrations used in our study are closer in line to measured concentrations from human liver samples (Olsen et al. 2003, Gallo et al. 2012). Although, the Warburg effect of cancerous cell lines cannot be ignored, despite that HepG2 cells are thought to be relatively metabolically similar to normal hepatocytes and our cells were acclimated to fatty acid oxidation conditions by removing glucose from the medium twenty-four hours prior to the experiment (Counihan et al. 2017, Arzumaninan et al. 2021). Studies using primary cell lines may be necessary to reveal a more definitive picture of PFOS’ effects on fatty acid metabolism. Taken together, inhibition of β-oxidation may not be a primary mechanism by which PFOS facilitates steatosis, and hepatocytes may be simply overwhelmed by triglycerides produced from alternative sources.

An abundance of energy sources is one of many factors that positively influences SRC (Marchetti et al. 2020). Both pyruvate and fatty acids produce acetyl-CoA that feeds into the Krebs cycle for
later ATP generation in the mitochondria (Marchetti et al. 2020). PFOS exhibited no effect on pyruvate concentrations in HepG2 cells but drastically increased triglyceride concentrations when the medium was supplemented with oleic acid (Figure 2.4). Oleic acid supplementation is a common mechanism used to study NAFLD in vitro (Xie et al. 2016, Guo et al. 2020). The increase in triglyceride concentration coupled with no changes in palmitate oxidation may indicate that increased fatty acid metabolism may not be the mechanism underlying the increased OCR observed during the Mitostress test and that triglycerides may simply be accumulating, contributing to the steatosis associated with PFOS exposure (Das et al. 2017, Hamilton et al. 2021). Importantly, this provides further evidence for the steatotic effects of PFOS in humans.

Glycolytic reserve was increased following treatment with 0.2 µM PFOS (Figure 2.3), however, pyruvate was not quantified in our HepG2 cells following treatment with 0.2 µM PFOS. Glycolytic reserve is measured following inhibition of ATP synthase (complex V) with oligomycin, forcing the cells to utilize glycolysis for energy production (Gleeson et al. 2018). Pyruvate quantification following low concentration PFOS exposure may provide a partial explanation for the increases observed in glycolytic reserve.

Activation of PPARα has been a major hypothesis suggested to explain the effects of PFOS on NAFLD in rodents (Das et al. 2017). However, our lab observed no changes in mRNA expression in HepG2 cells of genes commonly regulated by PPARα or other subtypes such as ACOX, CD36, CPT1a or ANGPTL4 (Figure 2.5). PPARγ expression trended upwards, but the difference was not statistically significant (Figure 2.5). No changes were observed in other metabolically important genes such as HMGCR or HKII (Figure 2.5). Interestingly, SCD1 expression was downregulated
in a dose dependent manner (Figure 2.5). This was unexpected, as SCD1 is critically involved in monounsaturated fatty acid (MUFA) production, and its expression is inversely associated with steatosis (Zhu et al. 2019).

Most importantly, SREBF1c, FASN and FoxA2 mRNA expression was upregulated following treatment with 1 µM PFOS for 24 hours (Figure 2.5). FoxA2 is a transcription factor that activates expression of genes involved in β-oxidation of fatty acids and is associated with increased metabolism and reduced adiposity and hyperlipidemia (Wolfrum and Stoffel 2006). Expression of FoxA2 is increased in NAFLD, potentially due to activation by excess fatty acids acting in a negative-feedback manner (Gosmain et al. 2005, Heintz et al. 2019). SREBF1 is one of the key transcription factors regulating expression of lipogenic genes such as FASN through sterol response element (SRE) binding and is determined to be a necessary component in facilitating insulin-mediated lipogenesis as it is positively regulated by insulin receptor substrate 1 (IRS1) (Gosmain et al. 2005, Kohjima et al. 2008). Furthermore, both IRS1 and SREBF1 expression is upregulated in NAFLD cases, and lipogenesis is quickly becoming recognized as a key contributor of lipid accumulation in steatosis (Kohjima et al. 2008, Ferre and Foufelle 2010).

Lipogenesis has high energy requirements and is coupled to glucose metabolism as an energy source via insulin-SREBF1c signaling (Ferre et al. 2021). It is important to note that all glycolytic parameters measured in addition to SREBF1c, FASN and FoxA2 expression were increased following treatment with lower concentrations of PFOS. Additionally, glutamine has also become recognized as a potentially remarkable substrate used as a precursor for fatty acid synthesis, including synthesis controlled by SREBF signaling (Metallo et al. 2012, Guo et al. 2014, Wallace
Taken together, the potential effect of PFOS on lipogenesis in hepatocytes provides an additional hypothesis for PFOS-mediated NAFLD and should be further investigated. Although, it should be noted that the gene expression and metabolic analysis was performed under slightly different conditions, without the presence of oleic acid. Future studies should aim to reconcile these differences.

AMPK is a key regulator of energy status within the cell and acts antagonistically to insulin by inhibiting lipogenesis and its mediators such as SREBF1 or mTOR proteins (Hansmannel et al. 2006, Peterson et al. 2011, Herzig and Shaw 2018). We unexpectedly observed an increase in AMPK activation in HepG2 cells following treatment with 5 µM PFOS for 60 minutes (Figure 2.6). However, this experiment was not performed using a lower concentration of PFOS, which may have resulted in a different outcome. This finding also disagrees with a recent study that demonstrated decreased AMPK phosphorylation in PFOS-treated HepG2 cells (Salter et al. 2021). This experiment was performed using 25µM PFOS, and AMPK activation was measured 10 hours after PFOS treatment (Salter et al. 2021). The effects of PFOS on AMPK activation is likely scenario dependent with respect to factors such as concentration and length of exposure.

PFOS repressed basal and ATP-production coupled respiration which subsequently increased SRC due to no significant changes in maximal respiration (Figure 2.7). PFOS also decreased proton leak correlating with decreased metabolic activity (Figure 2.7) (Jastroch et al. 2008). The role of skeletal muscle in the maintenance of organismal metabolic homeostasis is highly dependent on its sensitivity to insulin, which can be altered by a number of factors including decreased mitochondrial activity (Stump et al. 2009, Yazici and Sezer 2017). Thus, disruption in skeletal
muscle metabolism can be detrimental to other tissues as well, contributing to metabolic syndrome (MS) (Stump et al. 2009). This additionally correlates with our previous transactivation data indicating PFOS inhibits PPARδ and provides data suggesting skeletal muscle represents a serious target for PFOS toxicity (Eccles, 2022).

Finally, C2C12 cells were much more sensitive to PFOS treatment compared to HepG2 cells, qualitatively (Figures 2.1 and 2.7). HepG2 cells have been previously described as resistant to PFOS due potentially to low levels of OATP1B1 and 1B3 transporters which are primarily responsible for PFOS uptake into the liver (Godoy et al. 2013, Salter et al. 2021). This should be considered when interpreting all data from this study and suggest the effects of PFOS on triglyceride accumulation and lipogenic gene expression may be potent even at very small concentrations.
**Conclusion**

PFOS induces steatosis and expression of lipogenic genes in HepG2 cells with slight increases in overall and glycolysis-specific parameters of metabolism. Importantly, no effects on palmitate metabolism in HepG2 cells were observed following PFOS treatment, nor effects on gene expression regulated by PPAR signaling. This study provides data to support an alternative hypothesis by which PFOS contributes to hepatic steatosis through increased lipogenesis. Future studies should further investigate the effects of PFOS on SREBP1-FASN signaling and their subsequent effects on lipid accumulation.
References


Chapter 3: CYP2B6-derived Oxylipins Increase Triglyceride Concentrations and Perturb Gene Expression Associated with Fatty Acid Uptake and Synthesis in HepG2 Cells

Abstract

The prevalence of Non-Alcoholic Fatty Liver Disease (NAFLD) is quickly rising and can lead to detrimental health outcomes such as Non-Alcoholic Steatohepatitis (NASH), liver failure, and cancer. Recent studies have indicated that cytochrome P450 2B6 (CYP2B6) is an anti-obesity CYP in humans and mice. Cyp2b-null mice are high-fat diet induced obese and human CYP2B-transgenic (hCYP2B6-Tg) mice reverse the obesity and diabetes. However, CYP2B6 expression does not come without complications as the male mice also exhibit increased hepatic steatosis (fatty liver) in association with the increased presence of several oxylipins with 9-hydroxyoctadecadienoic acid (9-HODE) produced from linoleic acid (LA, 18:2, ω-6) the most prominent of these. In addition, CYP2B6 preferentially produces 9-hydroxyoctadecatrienoic acid (9-HOTre) in vitro from alpha-linolenic acid (ALA, 18:3, ω-3), but high-fat diets contain low amounts of ALA. Transactivation assays indicate that 9-HODE and 9-HOTre both activate PPARα and inhibit PPARγ. To test whether these oxylipins alter mitochondrial metabolism, Seahorse assays were performed to detect changes in the metabolic activity of HepG2 cells following 9-HODE or 9-HOTre treatment. 9-HODE exhibited no effect on metabolism. 9-HOTre increased spare respiratory capacity, slightly decreased palmitate metabolism, and increased non-glycolytic acidification in a dose dependent manner. Both compounds increased triglyceride concentrations in HepG2 cells when sustained with medium containing oleic acid, and both compounds increased pyruvate concentrations, most strongly by 9-HOTre; consistent with increased spare respiratory capacity. qPCR analysis revealed several perturbations in fatty acid uptake and metabolism gene expression in HepG2 cells. 9-HODE increased expression of cluster
of differentiation 36 (CD36), fatty acid synthase (FASN), PPARγ and the forkhead box protein A2 (FoxA2) that are involved in lipid uptake. 9-HOTrE decreased angiopoetin-like 4 (ANGPTL4) expression and increased FASN expression consistent with increased liver triglycerides. Our findings further support the hypothesis that 9-HODE and 9-HOTrE promote steatosis, but through different mechanisms as 9-HODE is directly involved in fatty acid uptake and synthesis; 9-HOTrE weakly inhibits mitochondrial fatty acid metabolism while increasing the utilization of glutamine.
Introduction
Non-alcoholic fatty liver disease (NAFLD) affects nearly 30% of Americans and is growing concern worldwide (Loomba and Sanyal 2013). Histologically diagnosed by the presence of large triglyceride droplets in greater than 5% of hepatocytes, NAFLD can progress from steatosis to more serious and less-reversible conditions such as non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (Loomba and Sanyal 2013, Friedman et al. 2018). Steatosis occurs through one of the following four canonical mechanisms: excessive hepatic lipid synthesis, increased hepatic uptake of fats, decreased hepatic export of fats, and decreased fat metabolism (Friedman et al. 2018). NAFLD often accompanies obesity which is largely a result of excessive calorie consumption and lack of exercise (Gross et al. 2017). However, the threat of toxicant mediated NAFLD has become increasingly apparent (Rajak et al. 2022).

Cytochrome P450 2B6 (CYP2B6) is highly expressed in the liver and is a key metabolizer of pharmaceuticals, pesticides and endogenous ligands such as bile acids, steroids, and polyunsaturated fatty acids (PUFAs) (Hodgson and Rose 2007, Wang and Tompkins 2008, Olack et al. 2022). In addition to detoxification, recent data indicates Cyp2b enzymes such as human CYP2B6 function in lipid metabolism and play a role in obesity and NAFLD. CYP2B6 expression is inversely proportional to Body Mass Index (BMI) in humans and is the only detoxification CYP that is inversely correlated with obesity with an association between low levels and obesity (Krogstad et al. 2020). Cyp2b’s are highly induced by a high-fat diet (Hoek-van den Hil et al. 2015, Heintz et al. 2022) and Cyp2b-null mice are diet-induced obese with NAFLD in males (Damiri and Baldwin 2018, Heintz et al. 2019).
However, the addition of human CYP2B6 to Cyp2b-null mice (hCYP2B6-Tg mice) reduced obesity and diabetes as expected but increased fatty liver disease (Heintz et al. 2022). CYP2B enzymes are sexually dimorphic (Lamba et al. 2003, Kumar et al. 2017) as are several of the transcription factors that regulate their expression (Hashita et al. 2008, Kumar et al. 2017). The role of murine Cyp2b members or human CYP2B6 may differ in their role protecting against or promoting steatosis, or they may be affected by factors such as diet composition and sex (Heintz et al. 2019, Heintz et al. 2020). Furthermore, the emerging toxicant, perfluorooctane sulfonate (PFOS) induces CYP2B6 and increases steatosis, where the enzyme’s role in promoting or protecting against NAFLD was again complicated by diet (Hamilton et al. 2021). CYP2B6 was protective of PFOS-mediated steatosis under a ND scenario, but exacerbated PFOS-mediated steatosis with PFOS/HFD-cotreatment (Hamilton et al. 2021). This data further implicates a role for CYP2B6 in metabolism and lipid homeostasis.

CYP2B6 is regulated by multiple transcription factors, some of which have known roles in energy metabolism (Dong et al. 2009, Deol et al. 2015). For example, the constitutive androstane receptor (CAR) regulates transcription of CYP2B6, and its activation improves metabolic diseases such as hyperglycemia, insulin resistance, and hepatic steatosis (Dong et al. 2009). The forkhead box protein A2 (FoxA2), which has a known function in insulin signaling, also regulates CYP2B6 and additionally controls expression of genes involved in fatty acid oxidation (Kohjima et al. 2005)

CYPs represent one of three major synthesis pathways for oxylipins, biologically potent signaling molecules oxidized from polyunsaturated fatty acids (PUFAs) (Barquissau et al. 2017, Eccles and Baldwin 2023). Oxylipins typically act in autocrine and paracrine fashion to regulate and affect
important physiological processes such as inflammation, chemotaxis and cellular differentiation (Dennis and Norris 2015, Gabbs et al 2015). Using CYP2B6-containing baculosomes, our laboratory further demonstrated that CYP2B6 produces several oxylipins at the 9th and 13th carbon atoms (Heintz et al. 2022). CYP2B6 preferentially metabolized linoleic acid (LA, 18:2, ω-6) to 9-hydroxyoctadecadienoic acid (9-HODE) and alpha-linolenic acid (ALA, 18:3, ω-3) to 9-hydroxyoctadecatrienoic acid (9-HOTrE), as well as produced other 9- and 13-oxidized PUFAs as hydroxy, ketone, or epoxides to a lesser degree (Heintz et al. 2022). While CYP2B6 exhibited higher affinity for ALA, metabolites of LA were present in far higher concentrations in the serum of HFD-fed hCYP2B6-TG mice (Heintz et al. 2022). Although both LA and ALA are components of a HFD, LA is approximately 20X more common and this difference in availability of substrate most likely caused the differences observed between in vivo and in vitro preferences of lipid (Poudyal et al. 2013, Whelan and Fritsche 2013, Greupner et al. 2018, Heintz et al. 2022).

The roles of 9-HODE and 9-HOTrE are not known and poorly studied. Although, 9-HODE is involved in several cellular and physiological processes as a potential mediator of inflammation following ischemic stroke, an inducer of fatty acid binding protein 4 (FABP4) via PPARγ activation, and is also positively associated with pain (Osthues and Sisigano 2019, Szczuko et al. 2020, Eccles and Baldwin 2023). Additionally, 9-HODE induces lipid uptake in monocytes as well as monocyte differentiation, highlighting its diverse functions (Quaranta et al. 2022). Furthermore, 9-HOTrE is even less studied, although it has been found to be downregulated in subcutaneous white adipose tissue (scWAT) in obese individuals (Fisk et al. 2022).
The peroxisome proliferator activated receptors (PPARs) are nuclear receptors primarily expressed in metabolically active tissue such as the liver, skeletal muscle and adipose, that regulate gene expression associated with fatty acid uptake and metabolism and are already implicated in metabolic diseases such as insulin resistance and steatosis (Haluzik and Haluzik 2006, Zhang et al. 2019, Xie et al. 2020, Eccles 2022). Given that natural ligands for the PPARs include fatty acids and their metabolites, our laboratory conducted transactivation assays to test whether a suite of CYP2B6-produced oxylipins activated the PPARs. 9-HODE and 9-HOTrE activate or inhibit the individual PPARs (α/δ/γ). Both oxylipins were some of the stronger activators of PPARα, the predominant isotype in the liver. They also inhibited PPARγ, the primary isotype expressed in adipose tissue (Eccles 2022), but earlier studies showed activation (Han et al. 2000, Itoh et al. 2008). Given their growing association with NAFLD and obesity, we hypothesize that the CYP2B6-derived oxylipins, 9-HODE and 9-HOTrE may influence hepatic energy metabolism through activation or inhibition of the PPARs that contribute to steatosis. The purpose of this study is to determine the effects of these oxylipins on lipid homeostasis in the liver through potential perturbation of fatty acid uptake, synthesis, and metabolism.
Materials and Methods

Cell Culture

HepG2 cells (Addex Bio, San Diego, CA) were cultured in Dulbecco’s Modified Eagle Medium (4.5 g/L glucose and sodium pyruvate, Corning, Corning, NY) consisting of 1% insulin-transferrin-selenium (ITS, Corning, Corning, NY), 1% penicillin-streptomycin (Gibco Cell Culture, Grand Island, NY), 1% L-glutamine (Gibco), and 10% fetal bovine serum (FBS, Cytiva USA, Marlborough, MA) at 37°C in a humidified atmosphere buffered with 5% CO₂ (Rungta et al. 2011).

Quantitative Real-Time PCR (qPCR)

HepG2 cells were seeded in 6-well plates, grown to complete confluency before extracting RNA, and were treated with 0, 1 or 5 µM 9-HODE or 9-HOTrE with DMSO as the carrier and included in the control group (n = 6). Twenty-four hours before RNA extraction. RNA extracts were obtained from the cells in each well using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Following sample purity assessment using a Nanodrop (Thermo Scientific, Waltham, MA), cDNA was synthesized from 2 µg of RNA with 5X RT buffer, dNTPs, random hexamers, and MMLV reverse transcriptase (Promega, Madison, WI) at 42°C for 60 minutes as described by us previously (Roling et al. 2004).

To measure gene expression, qPCR was performed with diluted cDNA, forward and reverse primers (Table 3.1), molecular grade water, and 0.25X SyBR Green Master Mix (Qiagen, Frederick, MD) on an iCycler (Bio-Rad, Hercules, CA) in triplicate. A standard curve was performed with 1:4 serial dilutions from 1:1 to 1:1024 to determine the efficiency of each reaction.
To ensure purity of the product, a melt curve was performed. Gene expression data was normalized to the geometric mean of 18S and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by using the inverted Muller’s method (Muller et al. 2002, Roling et al. 2004).

Table 3.1. qPCR primer information

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence (5' → 3')</th>
<th>Reverse Sequence (5' → 3')</th>
<th>Annealing Temperature (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>ACACCGAGGACTCTTTCGGA</td>
<td>GGAAGGGCAAGTCCCGATG</td>
<td>61.6</td>
</tr>
<tr>
<td>CD36</td>
<td>TCTTTCTGCGAACCAATG</td>
<td>AGCCTCTGTCTGCCAATCTG</td>
<td>54.2</td>
</tr>
<tr>
<td>ACOX</td>
<td>GGCCTATTGCAAGGACCT</td>
<td>AGGTGAAGCCTTTGACCTCCAGCC</td>
<td>60.3</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>GATGCGGAGAAGCTGCTTAT</td>
<td>GCTGTGTTGCAAGAAGCGCAA</td>
<td>61.0</td>
</tr>
<tr>
<td>FASN</td>
<td>TGCGTGGCCCTTTGAAATGTG</td>
<td>CTCATGTCGCTGAATGCT</td>
<td>57.9</td>
</tr>
<tr>
<td>PPARγ</td>
<td>AACCCCTTCACTACTTGGAC</td>
<td>CAGGCCCTCAGTTAGTG</td>
<td>55.0</td>
</tr>
<tr>
<td>HMCGR</td>
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<td>AGCCGAACAGCAGATGAT</td>
<td>55.2</td>
</tr>
<tr>
<td>Cpt1a</td>
<td>GATTTTCTGTGCTGGTCTTG</td>
<td>CTCCTCTGTGCAATGTA</td>
<td>55.8</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>GGACCACAAGCAGCTAGACCA</td>
<td>GATCCCCAAAACCCGCTT</td>
<td>60.8</td>
</tr>
<tr>
<td>SCD1</td>
<td>TCTAGCTCTATACCCACCACCA</td>
<td>TCCTGCTTACCTTACCTGCCCTC</td>
<td>59.6</td>
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<tr>
<td>FoxA2</td>
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<td>CTGTGTCATGCCGTCACTCC</td>
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<td>CCCGTGCCCACAATCGAG</td>
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<td>18S</td>
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<tr>
<td>GAPDH</td>
<td>CTTTCAAGCCTCCACTA</td>
<td>CTTGAAAGATGATGGG</td>
<td>50.0</td>
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</tbody>
</table>

Seahorse Assays

Mitostress assays (Agilent Technologies, Santa Clara, CA) were performed using a Seahorse XFe24 Analyzer (Agilent) to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of HepG2 cells. HepG2 cells were treated with 0.2, 1 or 5 µM 9-HODE or 9-HOTrE with DMSO included in the control group 24 hours prior to the Seahorse measurements (n = 5). Growth medium was exchanged for a substrate-limited assay medium containing Seahorse XF DMEM, 1 mM pyruvate, 2 mM glutamine and 10 mM glucose (Agilent)
just prior to the assay. Cells were incubated at 37°C in a humidified environment without CO₂ for one hour and measurements were taken according to the developer’s protocol (Agilent).

Palmitate oxidation assays were performed similarly to Mitostress assays as described above except cells were cultured in DMEM (without glucose and sodium pyruvate, Gibco) containing 10% FBS, 1% Pen-Strep, 1% L-Glut, 300 nM sodium palmitate, 0.5 mM L-carnitine, 0.24 mM fatty acid free bovine serum albumin (BSA), and 2.25 nM sodium chloride treated 0 or 5 µM 9-HODE or 9-HOTrE for 24 hours with DMSO included in the control group. Palmitate oxidation assay medium consisted of Seahorse XF DMEM supplemented with 300 nM palmitic acid, 0.5 mM L-carnitine, and 2 mM glucose (Agilent), and assays were performed according to manufacturer’s protocol (Agilent).

Glycolysis stress tests were performed using HepG2 cells cultured in regular DMEM as described above. Twenty-four hours prior to the assay, cells were treated with 0, 0.2, 1 or 5 µM 9-HODE or 9-HOTrE with DMSO included in the control group as the carrier (n = 5). Just prior to the assay, growth medium was swapped for a substrate-limited assay medium containing Seahorse XF Base Medium (Agilent Technologies, Santa Clara, CA) supplemented with 2 mM glutamine. Cells were incubated and the assay was performed according to manufacturer’s protocol (Agilent).

Triglyceride and Pyruvate Quantification

Triglycerides and pyruvate were extracted from HepG2 cells seeded in 100 mm x24 mm cell culture dishes and grown to complete confluence. Triglyceride quantification experiments were performed using regular DMEM as described above, or DMEM supplemented with 300 nM oleic
acid. Forty-eight hours prior to extraction, cells were treated with 1 or 5 µM 9-HODE or 9-HOTrE with DMSO included in the control group (n = 6). Triglycerides and pyruvate were extracted and quantified using colorimetric and fluorometric assays, respectively, according to each kit’s instructions (Cayman Chemical, Ann Arbor, MI).

*pAMPK ELISA*

To measure AMPK phosphorylation, a cell-based, fluorometric ELISA was performed (LS Bio, Seattle, WA) as described by the manufacturer. HepG2 cells were treated with 5 µM 9-HODE or 9-HOTrE 60 minutes prior to fixation with DMSO included in the control group (n = 6). Data was normalized to the total protein within each well. Total protein was quantified using a fluorometric protein stain provided in the assay kit.

*Statistics and Visual Preparation*

Graphs were prepared and all statistics were performed using GraphPad Prism 7.0 (San Diego, CA). A one-way analysis of variance (ANOVA) was performed to determine statistically significance between three or more groups followed by Fisher’s LSD as a post-hoc test. Student’s t-test was performed to determine statistically significant differences between two groups. Chemical structures were generated using ChemDraw 21.0.0 (PerkinElmer, Shelton, CT).
Results

*Metabolic analysis of HepG2 cells following 9-HODE or 9-HOTrE exposure:*

Rates of mitochondrial respiration and glycolysis can be determined by measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) that are a direct result of electron transport chain (ETC) utilization and lactic acid fermentation from pyruvate (Mookerjee et al. 2015, Shah-Simpson et al. 2016). To test whether 9-HODE and 9-HOTrE alter overall mitochondrial respiration, Seahorse mitostress assays were performed with HepG2 cells. No effect on any parameter of respiration was observed following 9-HODE treatment (Figure 3.1); however, 9-HOTrE significantly increased spare respiratory capacity (SRC) (Figure 3.2). SRC is defined as the maximal respiration minus the basal respiration and is influenced by the availability of energy sources upstream of mitochondrial metabolism such as triglycerides and pyruvate and is an indicator of metabolic flexibility that is positively correlated with mitochondrial activity (Marchetti et al. 2020).

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**Figure 3.1: 9-HODE exhibited minimal effect on overall mitochondrial metabolism.** Data from Seahorse mitostress test in HepG2 cells treated with 0, 0.2, 1 or 5 µM 9-HODE 24 hours prior to the assay. Data were analyzed via one-way ANOVA followed by Fisher’s LSD and are expressed as mean ± SEM (n = 5).
Disruption of fatty acid metabolism is a common mechanism of facilitating NAFLD (Deprince et al. 2020). To determine whether 9-HODE or 9-HOTrE inhibits or activates β-oxidation, palmitate-oxidation assays were performed using HepG2 cells.

Figure 3.2: 9-HOTrE increased Spare Respiratory Capacity in a dose-dependent manner. Data from Seahorse mitostress test in HepG2 cells treated with 0, 0.2, 1 or 5 µM 9-HOTrE 24 hours prior to the assay. Data were analyzed via one-way ANOVA followed by Fisher’s LSD and are expressed as mean ± SEM. *p<0.05 (n = 5).

Figure 3.3: 9-HODE exhibited no effect on palmitate oxidation. Data from Seahorse substrate oxidation assay in HepG2 cells treated with 0 or 5 µM 9-HODE 24 hours prior to the assay. Data were analyzed via one-way ANOVA followed by Fisher’s LSD and is expressed as mean ± SEM. ****p<0.0001 (n = 5).
Similar to the mitostress test results, 9-HODE showed no effect on palmitate oxidation (Figure 3.3). 9-HOTrE decreased palmitate oxidation as a decrease in OCR was observed following injection of etomoxir, an inhibitor of Cpt1a (Figure 3.4). However, DMSO and 9-HOTrE treated cells exhibited similar maximal respiration (Figure 3.4). This data suggests that 9-HOTrE may induce a metabolic shift away from fatty acid metabolism, rather than reducing the cells’ ability to metabolize fatty acids.

To test whether 9-HODE or 9-HOTrE alter glycolysis, glycolytic stress tests were performed using HepG2 cells. No effect on any parameter of glycolysis was observed following 9-HODE treatment (Figure 3.5). Taken with the mitostress and palmitate oxidation tests results, these data suggest 9-
9-HODE likely has no effect on mitochondrial function. In the absence of glucose but presence of glutamine, 9-HOTrE increased non-glycolytic acidification in a dose dependent manner, indicating 9-HOTrE exposed HepG2 cells may be increasing the metabolism of glutamine slightly (Figure 3.6) (Pavlou et al. 2017).

**Figure 3.5: 9-HODE exhibited no effect on glycolysis as measured by glycolysis stress test.** Data from Seahorse glycolysis stress test in HepG2 cells treated with 0, 0.2, 1 or 5 µM 9-HODE 24 hours prior to the assay. Data were analyzed via one-way ANOVA followed by Fisher’s LSD and are expressed as mean ± SEM (n = 5).
Quantification of triglycerides and pyruvate in HepG2 cells following 9-HODE or 9-HOTrE treatment:

Hepatic steatosis is characterized by excessive triglyceride accumulation in the liver (Browning and Horton 2004). In addition, Acetyl-CoA can be derived from both triglycerides and pyruvate through oxidation reactions and increases mitochondrial OCR through citric acid cycle progression and formation of NADH and FADH₂, which facilitate electron transport chain (ETC) progression (Marchetti et al. 2020). This experiment was repeated to measure triglyceride concentrations in the presence of oleic acid, a commonly used method to investigate NAFLD in HepG2 cells because the media contains few lipids (Xie et al. 2016, Guo et al. 2020).
Triglycerides and pyruvate concentrations were measured from HepG2 cells following 48-hour treatment with 0, 1 or 5 µM 9-HODE or 9-HOTrE to test whether 9-HODE or 9-HOTrE may contribute towards steatosis as well as determine potential mechanisms by which 9-HOTrE affects OCR. Increased macromolecular energy sources such as pyruvate or triglycerides is also a likely mechanism by which spare respiratory capacity is increased (ref).

9-HODE and 9-HOTrE increased triglycerides at both concentrations in the presence of oleic acid (Figure 3.7). Without oleic acid, 9-HODE decreased triglycerides at 5 µM (Figure 3.7). 9-HOTrE increased pyruvate concentrations in a dose dependent manner (Figure 3.7). Although 9-HODE

Figure 3.7: 9-HODE (A-C) and 9-HOTrE (D-F) alter triglyceride and pyruvate concentrations in HepG2 cells under different scenarios. 9-HODE decreased triglycerides in HepG2 cells in a dose dependent manner (A) but increased triglycerides in the presence of oleic acid (B). 9-HOTrE increased triglycerides only in the presence of oleic acid (E). Both compounds increased pyruvate concentrations (C&F). Data were analyzed via one-way ANOVA followed by Fisher’s LSD and are expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (n = 6).
increased pyruvate at both concentrations, the strongest increase was observed following 1\(\mu\)M treatment (Figure 3.7). These results suggest both oxylipins facilitate triglyceride accumulation in HepG2 cells and further implicate CYP2B6 in NAFLD through its production of oxylipins (ref – Heintz 2022). Additionally, the increase in triglycerides and pyruvate by 9-HOTrE may potentially explain the increase in OCR via spare respiratory capacity by 9-HOTrE (Marchetti et al. 2020).

Quantitative real-time PCR (qPCR):
9-HODE and 9-HOTrE are ligands for the peroxisome proliferator-activated receptors (PPARs) specifically PPAR\(\alpha\) and PPAR\(\gamma\), nuclear receptors that are important regulators of fatty acid uptake and metabolism and are implicated in NAFLD (Zhang et al. 2019, Heintz et al., 2022). Furthermore, SREBF1 is a key transcription factor that regulates genes necessary for lipogenesis and is also implicated in steatosis (Kohjima et al. 2007). To determine potential molecular mechanisms by which 9-HODE and 9-HOTrE alter mitochondrial metabolism and contribute to steatosis, qPCR was performed using mRNA extracted from HepG2 cells following treatment with 0, 1 or 5 \(\mu\)M 9-HODE or 9-HOTrE for 24 hours. Typical biomarker genes of PPAR and SREBF1C activation were measured as well as a few other key metabolic genes.

PPAR\(\alpha\) is the primary PPAR subtype expressed in the liver and regulates genes that code for proteins important for fatty acid uptake and metabolism such as acyl-coenzyme A oxidase 1 (ACOX), carnitine palmitoyltransferase 1A (Cpt1a), cluster of differentiation 36 (CD36), and angiopoetin-like 4 protein (ANGPTL4) (Haluzik and Haluzik 2006, Yoshida 2007, Rakhshanderoo et al. 2010, Dihingia et al. 2018, Wu et al. 2018, Todisco et al. 2022). 9-HODE increased CD36 expression in a dose dependent manner (Figure 3.8). 9-HOTrE decreased ANGPTL4 expression
following treatment with the lowest concentration of 1\(\mu\)M (Figure 3.9). However, these genes are shared targets of all PPARs (Yoshida 2007, Zhou et al. 2008).

SREBF1c positively regulates fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1), and HMG-CoA-reductase (HMGCR), highlighting its role in facilitating lipogenesis (Gosmain et al. 2005, Burg and Espenshade 2011, Mauvoisin and Mounier 2011). Although, HKII is also regulated by SREBF1 (Gosmain et al. 2005). FASN is also regulated by PPAR\(\gamma\) (Kawai et al. 2010, Ahmed et al. 2016). Insulin positively regulates SREBF1 while negatively regulating the forkhead box protein A2 (FoxA2), which controls expression of genes involved in fatty acid oxidation (Kohjima et al. 2007).

9-HODE upregulated FASN expression in a dose dependent manner in HepG2 cells, while an increase in FoxA2 and PPAR\(\gamma\) was observed following treatment with 1 \(\mu\)M 9-HODE treatment (Figure 3.8). The increase in FASN mirrored an upward trend in SREBF1c, although no statistical significance in SREBF1c expression was observed (Figure 3.8). A significant difference in FASN expression was also observed following treatment with the highest dose of 9-HOTrE (Figure 3.9).
Figure 3.8: 9-HODE perturbed expression of genes involved in fatty acid uptake and synthesis. Heatmap showing differentially expressed genes following increasing doses of 9-HODE compared to DMSO control (n=6). Data were normalized to the geometric mean of 18S and GAPDH and is expressed as log\(_2\) fold change relative to control. Data were analyzed via one-way ANOVA followed by Fisher’s LSD. *p < 0.05.
AMPK activation by 9-HODE and 9-HOTrE in HepG2 cells:

AMP-activated kinase is an important sensor and regulator of intracellular energy status (Hardie et al. 2012). Although activated by multiple mechanisms, it is commonly activated via phosphorylation during instances of high adenosine monophosphate (AMP) to adenosine triphosphate (ATP) ratio (AMP:ATP) within the cell (Hardie et al. 2012, Jeon 2016, Herzig and Shaw 2018). Activation of AMPK upregulates energy producing pathways and mechanisms within the cell such as glycolysis and mitochondrial biogenesis while downregulating energy-consuming processes such as lipogenesis (Viollet et al. 2009, Hardie et al. 2012, Herzig and Shaw 2018, Liu
et al. 2021). Given that both 9-HODE and 9-HOTrE altered overall mitochondrial respiration and expression of metabolically important genes, we tested whether 9-HODE or 9-HOTrE activated or repressed AMPK in HepG2 cells. Both compounds increased FASN expression, suggesting potential increased lipogenesis (Figures 3.8 and 3.9) (Gosmain et al. 2005). Thus, we predicted we would observe a decrease in AMPK activation. However, a significant increase in pAMPK was observed following treatment with 5 µM 9-HODE, and 9-HOTrE increased pAMPK with a significantly stronger effect (Figure 3.10).

**Figure 3.10: 9-HODE and 9-HOTrE increased AMPK phosphorylation.** Graph showing cell-based ELISA data following 60-minute incubation with 5 µM 9-HODE (A) or 9-HOTrE (B). Data were analyzed via one-way ANOVA followed by Fisher’s LSD and are expressed as mean ± SEM *p<0.05, ***p<0.001 (n = 5).
Discussion

Mitochondrial function is crucial in the maintenance of energy homeostasis (Friedman et al. 2018). Seahorse mitostress assays were performed using HepG2 cells treated with 9-HODE or 9-HOTrE to determine their potential to disrupt mitochondrial metabolism given their previous associations with steatosis (Figures 3.1 and 3.2) (Heintz et al. 2022). 9-HODE exhibited no effect on mitochondrial metabolism whereas 9-HOTrE increased SRC in a dose dependent manner (Figures 3.1 and 3.2). SRC is influenced by several factors which include mitochondrial health and number in addition to an abundance of upstream energy sources such as triglycerides or pyruvate (Marchetti et al. 2020).

9-HODE exhibited no effect on glycolysis or palmitate metabolism as measured by seahorse assays and does not appear to alter mitochondrial metabolism in HepG2 cells (Figures 3.3 and 3.5). The mechanism, if any, by which 9-HODE potentially facilitates steatosis likely occurs through increased fatty acid import, increased synthesis, or decreased export, as these represent the primary mechanisms behind NAFLD (Friedman et al. 2018).

9-HOTrE treated HepG2 cells exhibited decreased sensitivity to etomoxir, a Cpt1a inhibitor, under basal conditions but not when FCCP was injected into the assay (Figure 3.4). We initially interpreted this as a potential preference switch from fatty acid oxidation to glycolysis, however, the only glycolytic parameter that was altered was non-glycolytic acidification, which is measured with only glutamine in the cell culture medium as an energy substrate and was increased in a dose-dependent manner (Figure 3.6).
Glutamine is a highly versatile amino acid, that can feed into the Krebs cycle for energy production, gluconeogenesis or lipid synthesis through various processes and pathways (Altman et al. 2016, Shah and Wondisford 2020). This progression through the Krebs cycle produces CO$_2$ and carbonic acid as a biproduct that acidifies the medium (Mookerjee et al. 2015). Thus, 9-HOTrE may induce Krebs cycle progression coupled to a previously mentioned or perhaps different metabolic process.

Hepatic steatosis describes the excessive accumulation of triglycerides within the liver, and an abundance of triglycerides can also increase the cell’s SRC. 9-HODE increased triglyceride accumulation in HepG2 cells at both concentrations when the medium was supplemented with oleic acid, a common technique used to study NAFLD in vitro (Figure 3.7) but decreased triglyceride concentration in a dose-dependent manner without oleic acid supplementation (Xie et al. 2016, Guo et al. 2020). Coupled with no changes in metabolism, 9-HODE may also have a small function in facilitating lipid export that is infinitesimal in a more realistic NAFLD model. 9-HOTrE increased triglyceride accumulation in HepG2 cells only when supplemented with oleic acid (Figure 3.7). This increase observed following treatment with both 9-HODE and 9-HOTrE is in agreement with our previous data indicating CYP2B6’s role in facilitating steatosis (Heintz et al. 2022).

While treatment with both oxylipins increased pyruvate concentrations in HepG2 cells, the effects were noticeably stronger with a more dose-dependent trend following treatment with 9-HOTrE (Figure 3.7). However, the metabolic data indicates no alterations in either direction of glycolysis by 9-HOTrE (Figure 3.6), suggesting an alternative source. Pyruvate can be generated from
multiple substrates, but notably from the metabolism of alanine (Gray et al. 2014). More importantly, the liver metabolizes alanine to glutamine and pyruvate in conjunction with muscle tissue, a process known as the alanine cycle (Petersen et al. 2019). It is intuitive to believe that pyruvate would be the preferred source of energy metabolism for our HepG2 cells, unless 9-HOTrE, and to a lesser degree 9-HODE trigger the use of pyruvate as a substrate for an anabolic process. Pyruvate can contribute to both gluconeogenesis through its conversion to oxaloacetate and phosphoenolpyruvate by pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK), respectively, as well as fatty acid synthesis through citrate formation followed by export to the cytoplasm and subsequent conversion to oxaloacetate then Acetyl-CoA, the fatty acid synthesis precursor (Melkonian et al. 2019, Kiesel et al. 2021).

A more in-depth measurement and analysis or tracking of these important contributors to the Krebs cycle, pyruvate, glutamine, and alanine, may provide more insight into the metabolic changes that are occurring due to treatment with 9-HOTrE. It is also important to note that the Seahorse assays only provide an overall view of the cell’s energy metabolism, and if metabolism of one source were to decrease the same amount that the metabolism of another similar source increased, no change would be observed in the OCR or ECAR. Thus, it is not unreasonable to think that 9-HODE may also induce some shifts in the ratios of these metabolic substrates. However, it appears to be unlikely.

Neither 9-HODE nor 9-HOTrE altered expression of common PPARα-controlled genes except for the dose-dependent upregulation of CD36 by 9-HODE (Figure 3.8). Although, CD36 is a shared target of many nuclear receptors in addition to PPARα that include PPARγ, the pregnane-X-
receptor (PXR), the liver-X-receptor (Zhou et al. 2008, Suzuki et al. 2021). Treatment with 1 µM 9-HODE induced upregulated of PPARγ of FOXA2, and upregulation of FASN was observed in a dose-dependent manner (Figure 3.8). Activation of PPARγ also induces further transcription of PPARγ, and CD36 is commonly regulated by PPARγ, highlighting the receptor’s role in facilitating cellular uptake of fats (Zhou et al. 2008, Wakabayashi et al. 2009). Additionally, FASN is a well-known target gene of PPARγ, and PPARγ regulates PEPCK in promoting gluconeogenesis, potentially accumulating evidence for a PPARγ-mediated mechanism of altering metabolic homeostasis by 9-HODE (Floyd et al. 2008, Galbraith et al. 2018, Ma et al. 2021). However, this disagrees with our previous transactivation assay data indicating 9-HODE inhibits PPARγ (Eccles 2022). Finally, upregulation of FoxA2 has been observed in NAFLD patients as a potential negative-feedback mechanism (Gosmain et al. 2005, Heintz et al. 2019).

The only differences in gene expression observed following 9-HOTrE treatment was an increase in FASN expression in a dose-dependent manner and a decrease in ANGPTL4 following treatment with 1 micromolar 9-HOTrE (Figure 3.9). This data provides a putative mechanism for the increases observed in triglycerides following treatment of HepG2 cells (Figures 3.2, 3.4, 3.6, and 3.7). Furthermore, this potentially indicates increased fatty acid synthesis from a less traditional source such as glutamine, as the more canonical, SREBF-FASN pathway did not appear to be activated, although SREBF1c is also regulated post-transcriptionally (Gosmain et al. 2005).
Both oxylipins increased AMPK activation in HepG2 cells following incubation for 60 minutes (Figure 3.10). This was unexpected as upregulation in FASN, a gene necessary for facilitating an energy-rich, anabolic process, was observed (Figures 3.8 and 3.9). AMPK activation inhibits insulin-mediated lipogenesis via inhibition of FASN among other contributing genes (Hansmannel et al. 2006, Peterson et al. 2011, Herzig and Shaw 2018). However, mRNA was extracted from our HepG2 cells following incubation for 24 hours, measuring a more long-term response than that of our AMPK assay.

A large body of research has investigated the functions of oxylipins in other cellular and physiological functions, however, this is the first study to our knowledge to investigate a deep role of oxylipins, and specifically CYP-produced in regulating energy metabolism in the mitochondria (Eccles and Baldwin 2023). Although CYPs may be tertiary producers of oxylipins in relation to
COX and LOX enzymes, this study provided evidence for significant roles of these oxylipins in cellular metabolism, contributing to steatosis (Eccles and Baldwin 2023).

One limitation of this study is the inconsistency of conditions between the triglyceride quantifications, performed with oleic acid supplementation, and the metabolic and gene expression experiments performed without oleic acid present. This would appear to be most relevant to the fatty acid oxidation assays, as the results may have differed due to a greater concentration of fatty acids within the cell induced with help from oleic acid, however, no differences were observed in 9-HODE treated cells despite an abundance of palmitic acid being provided to the cells (Figure 3.3). This data further suggests that the effects of 9-HODE on hepatic steatosis likely lie outside of the mitochondria, a contrast to those of 9-HOTrE. Future studies should determine the role of CD36-mediated steatosis induced by 9-HODE, while analyzing or tracking specific metabolic intermediates that are potentially altered in mitochondrial metabolism by 9-HOTrE.
Conclusion

Previously, CYP2B6 was protective of HFD-mediated weight gain, although accompanied by an increase in liver triglycerides. Both CYP2B6-produced oxylipins, 9-HODE and 9-HOTrE increased triglyceride accumulation in HepG2 cells with contrasting effects on overall energy metabolism in the mitochondria. While 9-HOTrE increased specific parameters of overall mitochondrial metabolism, 9-HODE exhibited no effect in OCR or ECAR, and perturbed gene expression differentially from 9-HOTrE, with induction of CD36, being arguably the most notable result. Our findings further implicate both 9-HODE and 9-HOTrE in HFD-mediated hepatic steatosis while providing differing potential mechanisms.
References


Chapter 4: Conclusion

All three compounds examined during this study, perfluorooctanesulfonate (PFOS), 9-hydroxyoctadecadienoic acid (9-HODE) and 9-hydroxyoctadecatrienoic acid (9-HOTrE) induced triglyceride accumulation in HepG2 cells, correlating with previous data generated from our laboratory as well as others associating these compounds with hepatic steatosis (Figures 2.4 and 3.7) (Hamilton et al. 2021, Heintz et al. 2022). While this is not surprising, overall, these compounds failed to alter mitochondrial respiration in any dramatic way, except for the effects of PFOS observed in C2C12 cells (Figure 2.12). Particularly, 9-HODE did not appear to affect mitochondrial respiration at all (Figure 3.1). The appreciable variety of mitochondrial responses to these compounds that were observed is reflective of the variation in gene regulation induced by these compounds as well.

Additionally, the stark contrast of responses observed in some cases at different doses or between different conditions, for example, is notable and naturally lends itself to further questions regarding modeling, kinetics, or even experimental design. Similar (although slightly stronger) mitostress test results were observed when PFOS was present in the assay media compared to when the cells were washed prior to the assay (data not shown). PFOS is incredibly lipophilic and has been shown to disrupt cellular surfaces such as cell and mitochondrial membranes, organelles, in addition to its known function as a surfactant (Hu et al. 2003, Vecitis et al. 2008). Downregulation of OATP1B1 and 1B3 is a characteristic of HepG2 cells that decreases their sensitivity to PFOS (Godoy et al. 2013, Salter et al. 2021). While our experiments utilized realistic PFOS concentrations, the amount of PFOS that enters the cells or remains free to alter cellular processes may be even lower (Olsen et al. 2003, Gallo et al. 2012). Further studies investigating PFOS uptake
kinetics or localization in HepG2 cells may help increase their authenticity as a model for mimicking exposure in humans.

9-HODE exhibited the least effect on the mitochondria in HepG2 cells (Figures 3.1, 3.3, and 3.5). However, 9-HODE dysregulated gene expression associated with peroxisome proliferator activated receptors (PPARs) and fatty acid metabolism the strongest of the oxylipins (Figures 3.8, 3.9, 3.10 and 3.11). This was not without seemingly inconsistencies as 9-HODE not only increased pyruvate concentrations in HepG2 cells despite no increase in glycolysis but most strongly increased pyruvate concentrations following treatment with a lower concentration (Figures 3.5 and 3.7). Additionally, PPARγ mRNA expression was upregulated following treatment with the lowest dose of 9-HODE, and further upregulated gene expression of CD36, commonly controlled by PPARγ, despite our previous data indicating 9-HODE as an inhibitor of PPARγ (Figures 3.8 and 3.10) (Zhou et al. 2008, Wakabayashi et al. 2009, Heintz et al. 2022).

9-HOTrE similarly increased pyruvate concentrations with no alterations in glycolysis except for non-glycolytic acidification (Figures 3.6 and 3.7). While this data potentially supports the increase in spare respiratory capacity (SRC) via increased glutamine metabolism, this does not explain the increase in pyruvate concentrations (Figures 3.2 and 3.7). A decrease in ANGPTL4 expression was observed following treatment with 1 µM 9-HOTrE, while an increase in FASN expression was observed following 5 µM 9-HOTrE, implying differential, dose-dependent effects (Figures 3.9 and 3.11). All three compounds only increased triglyceride concentrations when the medium was supplemented with oleic acid. Taken together, the mechanisms by which these compounds act
likely contain many intricacies and require specific questions to be asked with carefully and deliberately defined conditions for further investigation.

9-HODE, and to a lesser extent 9-HOTrE, has been a strongly implicated oxylipin from our recent studies investigating the role of CYP2B6-mediated steatosis (Heintz et al. 2022). However, the effects of 9-HODE appear to exclude the mitochondria, and its mode of promoting triglyceride uptake may occur through upregulation of the fatty acid transporter, CD36 (Figures 3.1 and 3.8). Repeating the triglyceride colorimetric assays utilizing an siRNA or another method to knockdown CD36 expression or pharmacologically inhibiting CD36 may further confirm its role in facilitating 9-HODE mediated steatosis. Although, the effects of increased FASN expression should not be ignored (Figure 3.10).

Compared to 9-HODE, 9-HOTrE altered mitochondrial metabolism considerably more, although its actions are less likely to occur through PPAR signaling (Figures 3.2, 3.9 and 3.11). Specifically, the increase in OCR during the mitostress may potentially be due to increased glutamine metabolism, as non-glycolytic acidification was also increased (Figures 3.2 and 3.6). Glutamine can support many processes within the cell such as energy production and fatty acid synthesis via metabolic conversion through the Krebs cycle (Altman et al. 2016, Shah and Wondisford 2020). Furthermore, FASN mRNA expression was upregulated by 9-HOTrE (Figure 3.11). This increase in FASN coupled with decreased fatty acid oxidation observed during the palmitate oxidation assay provides a putative mechanism by which 9-HOTrE facilitates triglyceride accumulation. During the recent high-fat diet (HFD) studies, CYP2B6 was associated with increased steatosis only under a HFD (Hamilton et al. 2021, Heintz et al. 2022). The mechanism by which CYP2B6
was protective of PFOS-mediated steatosis under a normal diet (ND) scenario likely does not involve the oxylipins, given the recent measured affinities of their parent polyunsaturated fatty acids (PUFAs), however, oxylipins are not necessarily reflective of the organism’s PUFA makeup, as their synthesis is highly regulated (Gabbs et al. 2015, Heintz et al. 2022, Olack et al. 2022). However, given their increasing association with steatosis, they may play a role in facilitating the exacerbated steatosis observed when hCYP2B6-Tg mice were co-treated with a HFD and PFOS (Hamilton et al. 2021).

Two of the primary hypotheses addressing PFOS-mediated steatosis include the inhibition of mitochondrial β-oxidation and inhibition of the PPARs (Das et al. 2017, Fragki et al. 2021). Energetically, PFOS exhibited little effect on the mitochondria in HepG2 cells, with most perturbations occurring during glycolysis (Figures 2.1 and 2.3). PFOS neither increased nor decreased palmitate oxidation, providing evidence against the inhibition of β-oxidation (Figure 2.2).

Little effect on gene expression associated with PPAR signaling was observed (Figures 2.5 and 2.6). A downregulation of SCD1 expression was also an unexpected finding, but more importantly, SREBF1 signaling pathway appeared to be more sensitive to disruption by PFOS compared to PPARs (Figures 2.5 and 2.6). SREBF1 has been called a “master regulator” of lipogenesis and has been shown to mediate the lipogenic effects of insulin through IRS1-SREBF1 signaling (Kohjima et al. 2008, Li et al. 2021). Furthermore, the role of SREBF1 signaling in NAFLD has becoming increasingly apparent, and PFOS has been associated with disruptions in the effects of caloric restriction’s effects on glucose and insulin tolerance (Salter et al. 2021, Kohjima et al. 2008).
SREBF1-FASN signaling promoting lipogenesis represents a putative mechanism of PFOS-mediated NAFLD in humans.
References


