PFOA Alters the Expression of Mitochondrial Metabolism Genes in Rats.

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Table of Contents

List of Tables page iii
List of Figures page iv
Chapter 1- Introduction page 1
Chapter 2- Mitochondrial Biogenesis page 3
  Rationale page 3
  Methods and Materials page 4
  Results page 7
  Figures page 10
  Tables page 15
Chapter 3- Urea Cycle Gene Expression page 17
  Rationale page 17
  Methods and Materials page 17
  Results page 19
  Figures page 22
  Tables page 27
Chapter 4- Discussion page 29
References page 34
<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primer sequences for real time PCR</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Primer sequences for real time PCR</td>
<td>27</td>
</tr>
</tbody>
</table>
### List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mtDNA copy number</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>PGC-1α mitochondrial biogenesis pathway</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>OXPHOS subunit gene expression</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>Protein expression images</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>mt biogenesis flow diagram</td>
<td>14</td>
</tr>
<tr>
<td>6</td>
<td>Coomassie stained SDS-PAGE gel</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>Urea cycle gene transcription</td>
<td>23</td>
</tr>
<tr>
<td>8</td>
<td>Protein expression images</td>
<td>24</td>
</tr>
<tr>
<td>9</td>
<td>Amino Acid catabolism gene transcription</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>Urea cycle flow diagram</td>
<td>26</td>
</tr>
</tbody>
</table>
Chapter 1- Introduction

Perfluoroalkyl acids (PFAAs) are compounds with a variety of industrial uses including: stain and water resistant coatings, fire fighting foams, and nonstick cookware. Long carbon chain PFAAs possess a high degree of biopersistence and have been detected in humans and wildlife all around the world (Hansen et al., 2001; Kannan et al., 2002a; Kannan et al., 2002b; Kannan et al., 2002c; Calafat et al., 2007), which has caused them to emerge as a popular subject of study in the toxicological community. One of the most common PFAAs, perfluorooctanoic acid (PFOA), has been shown in rodents to cause hepatomegaly, peroxisome proliferation, transactivation of peroxisome proliferator-activated receptors (PPARs), and increased mitochondrial DNA copy number (Ikeda et al., 1985; Sohlenius et al., 1992; Lake, 1995; Intrasuksri et al., 1998; Maloney and Waxman, 1999; Berthiaume and Wallace, 2002; Vanden Heuvel et al., 2006; Takacs and Abbott, 2007). In addition, rodents receiving PFOA display decreased weight gain (Griffith and Long, 1980), indicative of a metabolic imbalance.

Mitochondria are integral organelles of metabolism, thus the reported changes in mitochondrial biogenesis and weight gain highlight the need for further study of the effect of PFOA on mitochondria. Mitochondria possess their own genome with 13 of the more than 80 proteins of the electron transport chain (ETC) being encoded in mtDNA, with the rest being encoded in the nucleus (Ryan and Hoogenraad, 2007). As electrons are shuttled along the ETC, protons are pumped from the matrix into the intermembrane space, resulting in an electrochemical gradient that provides energy for ATP synthesis through ATP synthase. Previous work demonstrated that PFOA disrupts ATP generation
by increasing the permeability of the inner mitochondrial membrane (Starkov and Wallace, 2002; O'Brien and Wallace, 2004). Over time, such a disruption results in a bioenergetic imbalance, and to correct this imbalance the cell will induce mitochondrial biogenesis (Berthiaume and Wallace, 2002).

Peroxisome proliferator-activated receptor \( \gamma \) coactivator 1-\( \alpha \) (Pgc-1\( \alpha \)), is considered the master regulator of mitochondrial biogenesis (Kelly and Scarpulla, 2004). Bioenergetic imbalances, such as when AMP/ATP ratios are high, stimulate increased expression of Pgc-1\( \alpha \) (Puigserver et al., 1998; Baar et al., 2002). This in turn leads to increased expression of nuclear respiratory factors 1 and 2 (Nrf1 and Nrf2), both of which induce expression of nuclear encoded mitochondrial proteins (Ryan and Hoogenraad, 2007). Nrf2 also upregulates the expression of mitochondrial transcription factor A (Tfam), which is required for replication and transcription of mtDNA (Parisi and Clayton, 1991; Ryan and Hoogenraad, 2007). Because PFOA increases mtDNA copy number and disrupts ATP production, we hypothesized that PFOA treatment in rats stimulates mitochondrial biogenesis via the Pgc-1\( \alpha \) pathway.

Mitochondria also play a significant role in ureogenesis in mammals. Protein catabolism produces ammonia, a toxic molecule that can cause severe neurological damage or death if not properly removed from the blood. The urea cycle is a series of reactions that catalyzes the conversion of ammonia to urea and is composed of five enzymes: carbamoyl phosphate synthase (CPS1), ornithine transcarbamylase (OTC), argininosuccinate synthase (ASS1), argininosuccinate lyase (ASL), and arginase (ARG1). CPS1 and OTC, the first two enzymes of the urea cycle, are located within the
mitochondria, while ASS1, ASL, and ARG1 are cytosolic. Expression of the urea cycle genes, with the exception of OTC, is coordinately regulated at the level of transcription (Morris, 1992). Conditions of starvation or a high protein diet stimulate urea cycle gene transcription through a signaling pathway that results in the phosphorylation of the cAMP response element binding protein (CREB) at serine 133 (Morris, 1992). It is believed this form of CREB then binds to CREB response elements (CREs) within the promoters of urea cycle genes and upregulates transcription. To date, CREs have been discovered for CPS1, ASS1, and ARG1 (Takiguchi et al., 1988; Schoneveld et al., 2007; Guei et al., 2008).

Western blot experiments using liver tissue from PFOA treated rats show a differential pattern of protein banding when visualized by Ponceau red staining. Through mass spectroscopy, a 150 kDa band that was decreased in the PFOA livers was identified as CPS1. In addition, Kersten et al. in 2001 showed that when PPARα was transactivated by WY-14,643, urea cycle gene expression was suppressed (Kersten et al., 2001). Because PFOA is a PPARα ligand and also leads to decreased CPS1 expression, we hypothesized that PFOA treatment in rats would alter urea cycle and amino acid catabolism expression.

Taken together, we intend to test the hypotheses that PFOA treatment alters two different aspects of mitochondrial metabolism: mitochondrial biogenesis and urea cycle gene expression.
Chapter 2- Mitochondrial Biogenesis

Rationale:

In rodents, PFOA treatment causes an increase in mtDNA copy number and also transactivates PPAR nuclear receptors (Berthiaume and Wallace, 2002; Vanden Heuvel et al., 2006). An increase in mtDNA is one of the products of mitochondrial biogenesis, which occurs through a pathway regulated by Pgc-1α, a coactivator known to interact with PPARs (Vega et al., 2000; Puigserver and Spiegelman, 2003). We hypothesized that PFOA treatment in rats stimulates mitochondrial biogenesis through the Pgc-1α pathway.

Materials and Methods:

Animals, dosing, and liver collection

Liver tissues were obtained from a study contracted by the 3M Company (St. Paul, MN). Adult male Sprague-Dawley rats (n=5 per treatment group) were subjected to a 12 hour light/dark cycle and provided SM R/M-Z rodent diet (SSNIFF Spezialdiaten GmbH, Soest, Germany) and tap water ad libitum. Control animals were given Milli-U water as vehicle and treated rats received ammonium PFOA at 30 mg/kg via oral gavage daily for 28 days. This dose was selected as a low range dose that causes a slight inhibition of body weight gain (data not shown). In all other regards the animals appeared healthy. Animals were euthanized and a portion of the midlobe region of livers from each of 5 rats per treatment group was stored separately in RNAlater (Applied Biosystems/Ambion, Austin, TX) at -80 °C; these tissues were used for all experiments.

Protein extraction and western blotting
100 mg of frozen liver tissue was thawed and cut into small sections and homogenized in 900 µl RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 0.5% Na deoxycholate, 0.1% SDS, 1% Igepal) using a 7 ml glass homogenizer. Debris from the homogenate was removed by centrifugation at 1000 x g for 10 minutes, and 10 µl per sample protease inhibitor cocktail P8340 (Sigma Aldrich Inc. St. Louis, MO.) was added prior to freezing. Protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL).

Samples were diluted in Laemmli buffer (4% SDS, 125 mM Tris, pH 6.8, 2% β-mercaptoethanol, 0.001% bromophenol blue, 30% glycerol) and boiled for 5 minutes prior to gel electrophoresis. Protein separation was accomplished on 4-20% gradient tris-glycine gels (NuSep, Lawrenceville, GA) at 150V until dye reached the bottom of the gel. Proteins were transferred to nitrocellulose membranes in transfer buffer (25 mM tris, 190 mM glycine, 20% methanol, 0.01% SDS) at 100V for 90 minutes. Ponceau red staining was utilized to confirm complete transfer and as verification of equal protein loading in each lane.

All washing steps were carried out in TBST buffer (0.9% NaCl, 50 mM Tris pH 8.0, 0.001% Tween 20); blocking buffer consisted of 5% dry milk in TBST. Primary antibody dilutions and sources were as follows: Tfam (1:400; Aviva Systems Biology, San Diego, CA), Pgc-1α and Cox II (1:1000 and 1:100, respectively; Santa Cruz Biotechnology, Santa Cruz, CA), Cox IV (1:4000; Molecular Probes, Eugene, OR), β-actin and Tubulin (1:300 and 1:1000; respectively; Abcam, Cambridge, MA), and GAPDH (1:1000; Millipore, Billerica, MA). Proteins were detected using SuperSignal

**DNA/RNA isolation and Real time PCR**

10-20 mg of frozen liver tissue was thawed and ground in a glass homogenizer followed by further homogenization in a 1 ml tuberculin syringe with a 27 gauge needle. RNA was isolated using the RNeasy Mini kit (Qiagen Inc. Valencia, CA), DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen), and cDNA was synthesized using random primers along with the Omniscript Reverse Transcription Kit (Qiagen). All primers (Table 1) were designed using PrimerQuest software (Integrated DNA Technologies, Inc. Coralville, IA). Real time PCR was carried out using FastStart SYBR Green I kit (Roche Diagnostics, Indianapolis, IN) with 10µl reaction volume and performed in a LightCycler (Roche Diagnostics). Samples were quantified using a serial dilution of a well characterized DNA standard, which provided a comparative measure to verify the integrity of the reaction. 18S ribosomal RNA was used to normalize gene expression. Real time PCR of control and treated samples for each gene was performed on the same run to control for potential run to run variability. Extracted RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.) to measure the absorbance at 260 nm ($A_{260}$). RNA quality and purity was assessed by observing a spectral scan with a single prominent $A_{260}$ peak and an $A_{260}/A_{280}$ ratio greater than 2. All RT-PCRs were performed at the same time using the same master mix. mtDNA copy number is calculated as the number of copies of mitochondrial encoded cytochrome b per copy of the nuclear gene pyruvate kinase. Fold change for each
transcript was calculated as follows: [(mean of mRNA copies/18s rRNA copy PFOA)/
(mean of mRNA copies/18s rRNA copy control)].

Statistical Analysis

Results are expressed as mean ± S.E. of five replications and the statistical comparisons
were performed using an unpaired Student’s \( t \)-test. \( P \) values of < 0.05 were judged to be
statistically significant. Treatment-related changes in DNA copy number or transcript
expression were judged by non-paired comparisons to control rats.

Results:

A treatment-related change was determined by comparing mtDNA copy number
of control versus PFOA treated rats. Consistent with previous findings (Berthiaume and
Wallace, 2002), real time PCR indicated that mtDNA copy number in rats receiving
PFOA was significantly increased, being approximately 1600 copies per cell versus 800
copies in livers from control rats (Figure 1).

The PFOA-dependent increase of mtDNA gene dose provided an indirect
indication of a stimulation of mitochondrial biogenesis. Using real time PCR, we
quantified changes in transcription for components of the Pgc-1\( \alpha \)-mediated mitochondrial
biogenesis pathway, including: \( Pgc-1\alpha, Err\alpha, Nrf1, Nrf2, \) and \( Tfam \). Transcription of
\( Pgc-1\alpha \) and \( Err\alpha \), the upstream components responsible for increasing expression of
transcription factors such as \( Nrf1 \) and \( Nrf2 \), were both significantly increased in the
PFOA-treated rats (Figure 2). The extent of induction was a 2.0 and 1.3 fold greater,
respectively, for PFOA-treated compared to control rats. \( Nrf1 \) and \( Nrf2 \), which promote
the expression of \( Tfam \) and electron transport chain subunits, both exhibited
approximately a 1.5 fold increase (Figure 2), and *Tfam*, which is required for mtDNA replication and transcription, was significantly increased by 2.2 fold in livers from PFOA-treated rats compared to controls (Figure 2).

The OXPHOS proteins are embedded in the inner mitochondrial membrane, and PFOA has been shown to alter the permeability of this membrane, thus disrupting ATP generation (Starkov and Wallace, 2002). Considering that the mitochondrial genome encodes for 13 of the more than 80 proteins comprising the electron transport chain, we investigated whether PFOA treatment affects the transcription of these genes and if so, whether subunits encoded by mtDNA are affected differentially from those encoded in the nucleus.

To investigate this, we selected two genes from the NADH dehydrogenase respiratory complex I (*Nd2* and *Ndufs8*) and two from the cytochrome oxidase complex IV (*Cox II* and *Cox IV*), with one gene from each complex being encoded by mtDNA and the other encoded by nuclear DNA. Real time PCR revealed a statistically significant induction in the transcription for all four genes, ranging from approximately 2 to 9 fold (Figure 3). In addition, the subunits encoded by mtDNA, *Cox II* and *Nd2*, were induced on average 3 to 4 times more than their corresponding nuclear DNA encoded counterparts (Figure 3).

To further substantiate these results, we investigated protein expression to determine if the changes in gene transcription are manifested at the translational level. Two components from the mitochondrial biogenesis pathway (PGC-1α and TFAM) and two from the OXPHOS system (COX II and COX IV) were selected and protein
expression was determined via western blotting. PGC-1α protein was increased in the livers of PFOA treated rats (Figure 4). In contrast, the amount of TFAM protein was not substantially changed, and expression of COX II and COX IV were both decreased in the PFOA samples (Figure 4). Interestingly, β-actin and GAPDH were both increased in the PFOA treated animals, whereas Tubulin was unchanged (Figure 4). In light of the treatment-related changes in what are considered conventional loading controls, we determined that it was not appropriate to attempt to quantitatively express the results from the western blots.
Figure 1- mtDNA copy number was assessed as the ratio of mitochondria-encoded cytochrome b DNA copies to nucleus-encoded pyruvate kinase DNA copies via real time PCR. Statistical analysis was performed using a two-tailed Student’s t-test, and expressed as mean ± SE, (n=5); asterisk (*) denotes $p < 0.05$. 
Figure 2 - Gene expression for transcripts of the Pgc-1α mitochondrial biogenesis pathway was assessed by real time PCR. Ratios of PFOA to control were as follows: Pgc-1α-2.0, Errα-1.3, Nrf1-1.4, Nrf2-1.5, and Tfam-2.2. Statistical analysis was performed using a two-tailed Student’s t-test, and expressed as mean ± SE, (n=5); asterisk (*) denotes p < 0.05.
Figure 3- Gene expression for selected OXPHOS subunits was assessed by real time PCR. Ratios of PFOA to control were as follows: Cox II-7.1, Cox IV-2.2, Nd2-9.4, Ndufs8-1.8. Statistical analysis was performed using a two-tailed Student’s t-test, and expressed as mean ± SE, (n=5); asterisk (*) denotes $p < 0.05$. 
Figure 4 - Protein expression for PGC-1α, TFAM, COX II, COX IV, β-actin, GAPDH, and Tubulin from rat liver homogenate was analyzed by western blotting. The symbols (+) and (−) represent PFOA and control, respectively (n=5), each lane represents one individual animal.
Figure 5- Flow diagram illustrating the Pgc-1α mitochondrial biogenesis pathway in rodents and possible modes of activation by PFOA. The pathway is intended to illustrate current understanding of the literature (Ryan and Hoogenraad, 2007). PFOA may stimulate Pgc-1α activity through either direct or indirect activation of PPAR nuclear receptors or via bioenergetic changes sensed by AMPK. Abbreviations: (PFOA)-perfluorooctanoic acid, (FFA)- free fatty acids, (PPAR)- peroxisome proliferator-activated receptor, (AMPK)- AMP-activated protein kinase, (Pgc-1α)- peroxisome proliferator-activated receptor γ coactivator-1α, (Errα)- estrogen-related receptor α, (Nrf1/2)- nuclear respiratory factors 1 and 2, and (Tfam)- mitochondrial transcription factor A.
### Table 1- Primer sequences for real time PCR.

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<td>Errα</td>
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Chapter 3- Urea Cycle Gene Expression

Rationale:

Previous SDS-PAGE experiments with extracted liver proteins from PFOA treated rats showed treatment dependent alterations of specific protein bands (Figure 6). One of these bands was identified by mass spectroscopy as CPS1, the first enzyme of the urea cycle. In addition, treatment in mice with the PPARα agonist, WY-14,643 caused a suppression in urea cycle and amino acid catabolism gene expression (Kersten et al., 2001). In light of the fact that PFOA is an agonist for PPARα, we hypothesized that rats administered PFOA would demonstrate decreased expression of multiple urea cycle genes.

Materials and Methods:

Animal care and dosing

Animal care and dosing were carried out as described previously (Walters et al., 2009). As part of a study contracted by the 3M Company, adult male Sprague-Dawley rats were treated with PFOA via oral gavage at a concentration of 30 mg/kg daily for 28 days. Liver tissue from these rats was stored in RNAlater (Applied Biosystems/Ambion, Austin, TX) at -80°C and was used for all experiments.

RNA isolation and real time PCR

Thawed liver tissue (10-20 mg) was ground in a glass homogenizer followed by additional homogenization in a 1 ml tuberculin syringe containing a 27 gauge needle. The RNeasy Mini Kit (Qiagen Inc., Valencia, CA) was used to isolate RNA and cDNA
was created using the Omniscript Reverse Transcription Kit (Qiagen Inc.) with random primers. PrimerQuest software (Integrated DNA Technologies, Inc., Coralville, IA) was used to design all primers (Table 2). For real time PCR, a 10 µl reaction using the FastStart SYBR Green I Kit (Roche Diagnostics, Indianapolis, IN) was analyzed with a LightCycler (Roche Diagnostics). 18S ribosomal RNA was used for normalization of gene expression and quantification was assessed using a serial dilution of a well characterized DNA standard. To calculate fold change, the following formula was used: (mean of mRNA copies/18S rRNA copy PFOA)/ (mean of mRNA copies/18S rRNA copy control).

Protein extraction and western blotting

100mg of liver tissue was homogenized in RIPA buffer (150mM NaCl, 50 mM Tris, pH 8.0, 0.5% Na deoxycholate, 0.1% SDS, and 1% Igepal) and centrifuged to remove cellular debris. To each sample, 10µl of protease inhibitor cocktail P8340 (Sigma-Aldrich Inc., St. Louis, MO) was added; protein concentration was determined by BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL).

Extracted proteins were diluted in Laemmli buffer (4% SDS, 125mM Tris, pH 6.8, 2% β-mercaptoethanol, 0.001% bromophenol blue, and 30% glycerol) then boiled for 5 min. These samples were then separated on 4-20% gradient Tris-glycine gels (NuSep) and transferred to nitrocellulose membranes at 100V for one and a half hours in transfer buffer (25mM Tris, 190 mM glycine, 20% methanol, and 0.01% SDS). Confirmation of protein transfer was determined by staining membranes with Ponceau red.
A TBST buffer was used for washing membranes (0.9% NaCl, 50 mM Tris, pH 8.0, and 0.001% Tween 20) and blocking buffers consisted of either 5% nonfat dry milk or 5% BSA in TBST, depending on antibody. Antibodies were used at the following dilutions: CPS1 (1:8000; Abcam, Cambridge, MA), OTC (1:800; Abcam), ASS1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), CREB (1:200; Santa Cruz), S133 phosphorylated CREB (1:1000; Abcam). SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific Inc.) was utilized for detection of proteins and images were taken on a FluorChem digital imager (Alpha Innotech Corp., San Leandro, CA).

Mass spectroscopy

Extracted proteins were run on 4-20% gradient Tris-glycine gels (NuSep, Lawrenceville, GA) and bands were visualized by Coomassie Blue staining. Bands of interest (at approximately 150 and 75 kDa) were excised from the gel and sent to the Center for Mass Spectroscopy and Proteomics at the University of Minnesota. Data was then analyzed using Scaffold 2 protein identification software.

Statistical analysis

The results are expressed as mean ± SE for five replications and statistical comparisons were carried out using an unpaired Student’s t-test; statistical significance was set at a p value of <0.05.

Results:

Mass Spectrometry
Ponceau stained nitrocellulose membranes with alternating lanes of control and PFOA treated samples produced a banding pattern in which a 150 kDa and a 75 kDa band were decreased and increased by PFOA treatment, respectively (Figure 6). Both bands were excised from a Coomassie stained SDS-PAGE gel, with the 150 kDa band removed from the control lane and the 75 kDa band was cut from a PFOA treated sample lane. Excised bands were sent to the Center for Mass Spectroscopy and Proteomics at the University of Minnesota and protein analysis software identified the 150 kDa band as CPS1 and the 75 kDa band as the peroxisomal bifunctional enzyme (EHHADH). EHHADH expression is well known to be induced by PFOA treatment (Ikeda et al., 1985), but CPS1 has to our knowledge not yet been implicated in any PFOA studies.

**Urea Cycle Gene Transcription**

The five genes of the urea cycle (Cps1, Otc, Ass1, Asl, Arg1) were analyzed by real time PCR. Gene transcription of the two mitochondrial components of the urea cycle was altered by PFOA treatment, as a decrease of 40% for Cps1 and an increase of 70% for Otc was observed (Figure 7). The gene encoding the other ATP-dependent step of the urea cycle, Ass1, was significantly reduced 60% by PFOA treatment (Figure 7). Asl transcription was also significantly decreased by 30%, while Arg1 mRNA was unchanged (Figure 7). These results are consistent with the well-characterized pattern of coordinate urea cycle gene transcription, including Otc as the exception.

**Urea Cycle Protein Expression**

Protein expression of the urea cycle enzymes CPS1, OTC, and ASS1 was determined by western blotting (Figure 8). Protein amounts were not quantified as the conventional
loading controls GAPDH and β-actin were altered by PFOA treatment. CPS1 and ASS1 protein were decreased in the PFOA treated samples, and interestingly, OTC, which is not usually coordinately expressed with the other urea cycle enzymes, was also decreased.

**CREB Protein Expression**

The amounts of the phosphorylated and unphosphorylated forms of CREB were investigated using western blots. The expression of serine 133 phosphorylated CREB, which is the transcriptionally active form, was increased by PFOA treatment (Figure 8). Additionally, unphosphorylated CREB protein was decreased in the PFOA samples (Figure 8). The observed increase in serine 133 phosphorylated CREB was unexpected given that transcription of the coordinately expressed urea cycle genes was decreased.

**Amino Acid catabolism**

The decreased urea cycle gene expression may be due to the structural similarity of PFOA to a fatty acid, as it may cause a shift away from protein metabolism to lipid metabolism. To investigate this, we analyzed the transcription of two genes encoding mitochondrial enzymes directly upstream of the urea cycle that are responsible for generating ammonia from amino acids, glutaminase 2 (Gls2) and glutamate dehydrogenase (Glud1). Gls2 transcription was significantly decreased in the PFOA treated rats, indicative of reduced glutamine catabolism (Figure 9). In addition, we observed a statistically significant increase in Glud1 gene transcription, which suggests an increased demand for glutamate catabolism (Figure 9).
Figure 6- Coomassie-stained SDS-PAGE gel with 10 µg protein loaded per lane. Mass spectroscopy identified the 150 kDa band as carbamoyl phosphate synthase 1 (CPS1) and the 75 kDa band as peroxisomal bifunctional enzyme (EHHADH). While CPS1 alteration by PFOA is a novel finding, EHHADH is a standard marker for peroxisome proliferation.
Figure 7- Transcription of urea cycle genes was measured by real time PCR. Data are expressed as mRNA copies/18S rRNA copy. Statistical analysis was performed using a two-tailed student’s t-test and expressed as mean ± SE, n=5, and (*) denotes $p < 0.05$. Genes analyzed were carbamoyl phosphate synthase 1 (Cps1), ornithine transcarbamylase (Otc), argininosuccinate synthetase (Ass1), argininosuccinate lyase (Asl), and arginase (Arg1).
Figure 8- Western blot images for selected urea cycle enzymes ASS1, CPS1, and OTC, and for CREB and phosphorylated CREB (serine 133). Proteins were extracted from the livers of control and PFOA treated rats.
Figure 9- Transcription of glutaminase (Gls2) and glutamate dehydrogenase (Glud1) genes was measured by real time PCR. Data are expressed as mRNA copies/18S rRNA copy. Statistical analysis was performed using a two-tailed student’s t-test and expressed as mean ± SE, n=5, and (*) denotes $p < 0.05$. 
Figure 10- 28 day PFOA treatment in rats leads to decreased weight gain and induces a starvation response. This response activates a signaling cascade that results in the phosphorylation of CREB at serine 133, which leads to increased urea cycle gene transcription. However, PFOA transactivates PPARα which supersedes transcription of urea cycle genes as part of a PPARα mediated shift to lipid catabolism away from protein catabolism. Abbreviations: PFOA (perfluorooctanoic acid), PPARα (peroxisome proliferator-activated receptor alpha), cAMP (cyclic adenosine monophosphate), PKA (protein kinase A), CREB (cAMP response element binding protein), CRE (CREB response element).
Table 2- Primer sequences for real time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Product Size</th>
<th>Accession Number</th>
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<tr>
<td>Arg1</td>
<td>F: 5’-atcgtgtacatggttgagat-3’&lt;br&gt;R: 5’-actttgccaatccacagctttgc-3’</td>
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<td>Asl</td>
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<td>NM_021577</td>
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<tr>
<td>Ass1</td>
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<td>83 bp</td>
<td>NM_013157</td>
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<tr>
<td>Cps1</td>
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<td>190 bp</td>
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<td>Glu2</td>
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<td>108 bp</td>
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<tr>
<td>Glud1</td>
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<td>199 bp</td>
<td>NM_012570</td>
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<tr>
<td>Otc</td>
<td>F: 5’-tcctgtcaacaagggacg-3’&lt;br&gt;R: 5’-tcagggctttctcagcttg-3’</td>
<td>103 bp</td>
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<td>R: 5’-cttggatggtgtagccgtttct-3’</td>
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Chapter 4- Discussion

Our results demonstrate that PFOA treatment for 28 days in rats results in significant changes in gene expression related to mitochondrial biogenesis and the urea cycle. For mitochondrial biogenesis, we observed an increase in transcription for components of the Pgc-1α pathway and also an increase in mtDNA copy number. Given that PPARs are coactivated by Pgc-1α (Puigserver et al., 1998; Vega et al., 2000) and transactivated by PFOA (Intrasuksri et al., 1998; Vanden Heuvel et al., 2006; Takacs and Abbott, 2007), our results are consistent with the action of PFOA upon PPARs as being responsible for these observed effects on gene expression (Figure 5). Additionally, PPAR ligands can induce mitochondrial biogenesis, as was shown in Cox 10 conditional knockout mice that, when given the PPAR panagonist bezafibrate, displayed restored OXPHOS capacity (Wenz et al., 2008).

Alternatively, it may be the ability of PFOA to affect mitochondrial bioenergetics, and not the activation of PPARs which is the primary mechanism for the upregulation of the Pgc-1α mitochondrial biogenesis pathway. Intracellular fatty acids could accumulate as a result of PFOA’s ability to impede mitochondrial metabolism (Starkov and Wallace, 2002; O'Brien and Wallace, 2004), and since fatty acids are ligands for PPARs, it may be such a transactivation by endogenous fatty acids that induces the Pgc-1α pathway (Figure 5).

Rats treated with PFOA experience decreased weight gain, indicative of a bioenergetic imbalance. Such an imbalance can result in increased AMP/ATP ratios,
which may then stimulate mitochondrial biogenesis through the energy sensor AMPK (Figure 5). AMPK has been shown to activate mitochondrial biogenesis by stimulating Pgc-1α to coactivate and increase transcription of mitochondrial proteins (Jager et al., 2007). It is possible the decrease of OXPHOS subunit protein we observed could lead to AMPK and Pgc-1α activation because the cell is not able to maintain ATP production. In support of this, a prior study of MELAS disease demonstrated that mitochondrial biogenesis was stimulated in part by decreased OXPHOS protein (Rossmanith et al., 2008).

The urea cycle gene expression changes we observed are most likely due to activation of PPARα by PFOA, and not via the CREB pathway. A study by Kersten et al. (Kersten et al., 2001) demonstrated that treatment of mice with the PPARα agonist WY-14,643 reduced transcription for Cps1, Ass1, and Asl while Arg1 was unchanged, and the authors also showed that these changes were PPARα dependent as the decreased transcription was observed in wild type mice but not in PPARα KO mice. These findings in wild type mice match our results with PFOA treated rats. In addition, Kersten et al. also showed decreased urea cycle enzyme protein expression, and changes in Gls2 and Glud1 mRNA that are also consistent with our observations. Because of the similarities in treatment duration (28 days vs. 21 days) and species (rat vs. mouse), we believe the comparison between our study and Kersten et al. 2001 is with strong merit.

When phosphorylated at serine 133, CREB becomes active on the promoters of urea cycle genes and stimulates an upregulation in transcription (Morris, 1992). Interestingly, we observed an increase in the amount of phosphorylated CREB, yet
transcription for the coordinately expressed urea cycle genes was decreased. This suggests that the action of PFOA on PPARα supersedes CREB S133 mediated urea cycle gene transcription (Figure 10). A suppression of this type was demonstrated in a recent paper by Scoditti et al. 2010 in which cells treated with the PPARγ agonist rosiglitazone resulted in decreased transcription of the CREB target gene cyclooxygenase-2 (COX-2). The authors demonstrated that the decrease in transcription was due to an interference with the CREB response element within the COX-2 promoter (Scoditti et al., 2010); PPARα may be interfering in the same manner in our PFOA treated rats. It is also a possibility that PPARγ, which PFOA is known to transactivate (Vanden Heuvel et al., 2006), could be responsible for our observed suppression of CREB mediated transcription.

We feel that the suppression of urea cycle gene expression is representative of a larger metabolic shift induced by PFOA which causes lipid metabolism to be prioritized over protein metabolism. The rats received repeated administration of PFOA, therefore the lipid metabolizing pathways mediated by PPARα would be continually activated while simultaneously inhibiting pathways of protein metabolism. Over time, accumulated suppression of urea cycle transcription would manifest as decreased urea cycle protein expression, which is consistent with our observations, and would eventually result in hyperammonemia if the amount of protein fell below the threshold required to remove ammonia from the blood.

It should be noted that the changes observed in PFOA treated rats do not necessarily reflect what the response in humans would be. The PFOA concentration in
the serum of the rats in our study has been suggested to be approximately four orders of magnitude greater than the mean PFOA serum concentrations normally observed in humans (Dr. J. Butenhoff, personal communication). Therefore, with the concentration of PFOA so much greater in experimental rats than humans, it is unlikely that either increased mitochondrial biogenesis or decreased urea cycle gene expression would be observed in humans exposed to PFOA.

In addition, the PPARα response in rodents is much different than that reported in humans, even if we were to assume comparable serum concentrations of PFOA. Unlike rodents, transactivation of PPARα in humans does not lead to the same toxicity profile as observed in rodents. For instance, the long clinical history with PPARα agonists such as the fibric acids which are prescribed to lower serum cholesterol and triglycerides in humans, demonstrates no indication of tumorigenesis or peroxisome proliferation (Klaunig et al., 2003). Experimentally, a humanized PPARα mouse was shown to be much less responsive to PFOA treatment as increased expression of the PPARα target genes peroxisomal thiolase, bifunctional enzyme, and Cyp4a10 was observed only in wild type mice (Nakamura et al., 2009). The difference between rodent and human PPARα is such that the United States Environmental Protection Agency’s proposed scientific policy in 2003 was that rodent-based studies of PPARα induced liver tumor formation are not translatable in assessing human health hazards (USEPA, 2003). Thus, if PFOA were transactivating PPARα in humans, it would not be expected to show the deleterious effects observed in the rodent studies. It is due to the large differences in PFOA serum concentrations and the differential effects on PPARα between rodents and
humans that we conclude the changes we observed would most likely not be present in humans and therefore it would not be appropriate to extrapolate our findings to PFOA being a human health risk.

It is clear from our results that PFOA treatment in rats alters two crucial pathways in which mitochondria play an important role: energy metabolism and ureogenesis. PFOA is similar in structure to an endogenous fatty acid, and it is this resemblance that causes PFOA to alter these pathways via transactivation of PPARs. While the stimulation of mitochondrial biogenesis may be a direct effect owing to PPAR transactivation combined with Pgc-1α coactivation or indirect effect resulting from disrupted energy metabolism, the alteration of urea cycle gene expression is most likely from PPARα transactivation. These novel findings provide further evidence of the toxicity of PFOA and make a valuable contribution to the continuing toxicological assessment of PFOA.
References:


