Title: Gestational exposure of mice to environmentally persistent free radicals leads to increased weight gain in offspring.

Short Title: Gestational EPFR Exposure and Weight Gain

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# Abstract

# Introduction

Obesity is a major global health concern and emerging data supports a role for environmental pollutants in the pathogenesis of obesity and its comorbidities. Indeed, several studies have linked the exposure to environmentally persistent free radicals (EPFR’s), compounds that result from the incomplete combustion of organic materials, to impaired metabolic health in humans (1–4) and animals (5–9). Specifically, cross-sectional studies of human subjects who are chronically exposed to combustion derived particulate matter have shown associations with type 2 diabetes and cardiovascular disease (1, 4, 3), whereas murine models of chronic particulate matter exposure indicate that pollutants lead to elevated adipose tissue inflammation and insulin resistance (6, 7, 9). Despite these associations, there is a gap in our understanding of how chronic exposure to EPFR’s causes metabolic health abnormalities.

Gestational and early-life exposure to combustion-derived particulate matter has been associated with an increased risk of obesity in humans (10–12). This association is supported by data obtained from animal studies, where the offspring of pregnant female mice, which have been exposed to diesel exhaust *in utero*, are predisposed to weight gain and increased body weight as adults (13). However, the mechanisms of how environmental pollutants cause pro-obesity fetal programming remain to be determined.

The mechanisms by which EPFR’s cause metabolic abnormalities are likely to be complex and multifactorial, however, one promising mechanism involves alterations to mitochondrial function. The mitochondria are responsible for oxidative cellular energy production, reactive oxygen species production and are the primary site of the antioxidant defense system. Thus, defects in mitochondrial metabolism can have profound effects on whole body metabolic health. Indeed, mitochondrial deficiencies and structural abnormalities have been observed in adipose tissue (Xu, 2011A, Xu, 2011B), vascular tissue (Westbrook, 2010) and cardiac muscle (Li ,2015) following exposure to EPFR’s. However, the effects of EPFR’s on skeletal muscle mitochondrial function remains to be determined. The importance of skeletal muscle mitochondrial metabolism for maintaining metabolic health is becoming well recognized (Hoeks & Schrauwen, 2012; Russell, 2014; Stephenson & Hawley, 2014), with deficits in muscle quality and function, particularly during early development (Brown, 2014), being closely linked to many different metabolic disease states (Russell, 2014). In this study, we have investigated the effects of *in utero* exposure to EPFR’s on growth, metabolism, energy utilization and skeletal muscle mitochondrial function in a mouse model of diet-induced obesity.

# Methods and Materials

## MCP230 Preparation and Treatment

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## Animal Housing and High Fat Diet

Mice were maintained in a 12h light/dark cycle room at constant temperature and humidity and allowed unrestricted access to food and water. At 10 weeks of age, mice were switched from chow to a high fat diet, consisting of 45% of calories from fat (Research Diets catalog D12451). One mouse, a MCP230 treated animal had a malocclusion and was removed from all analyses. The UTHSC Institutional Animal Care and Use Committee approved all mouse procedures.

## Metabolite Assays

Glucose was determined using an AccuCheck glucometer. Serum hormone levels were determined using a Bio-Plex pro mouse diabetes multiplex immunoassay, BioRad (#171-F7001M) following the manufacturer’s instructions.

## Body Composition and Metabolic Cages

Mice were weighed weekly at approximately ZT10. Body composition was determined non-invasively using an echo-MRI 100 at approximately ZT10. Food intake during the HFD phase was determined on a per-cage level by weighing the food on a weekly basis. For pre-HFD food intake, this was the sum of food eaten during the time in the metabolic cages, as determined by scaled feeder.

Energy expenditure, ambulatory locomotor activity and respiratory exchange ratios were determined in a home-cage style comprehensive laboratory animal monitoring system (Columbus Instruments). Mice were placed in the cages at approximately ZT10 and monitored for 3-4 days. Data from the first 6h were discarded as this was the approximate amount of time for the mice to become accustomed to their new single-caged environment. The Oxymax software provided by the vendor calculated the volumes of O2, CO2, the respiratory exchange ratio, the ambulatory x- and y-phase physical activity and the food consumption.

## Tissue Collection and Nucleic Acid Preparation

After the 12 week high fat diet phase, mice were fasted overnight and anesthetized using… Immediately after death, quadriceps muscles were quickly and carefully dissected out, cleared of any visible adipose and connective tissue and snap frozen in liquid N2. Nucleic acids were isolated from frozen quadriceps samples via Trizol extraction. Following careful and complete removal of the RNA-containing aqueous phase and its subsequent column purification (Life Technologies), genomic DNA extraction buffer (Tris base [1 M], sodium citrate dibasic trihydrate [50 mM], guanidine thiocyanate [4 M]) was added to the tubes containing the remaining interphase and infranatant. Tubes were shaken vigorously and centrifuged at 12,000 G at room temperature for 30 minutes. The aqueous phase was then collected and the genomic DNA precipitated in isopropanol. Samples were re-spun at 12,000 G at 4°C to pellet the DNA. The DNA pellet was then washed in 70% ethanol, re-spun and, after careful ethanol removal, re-suspended in 1x Tris-EDTA buffer. cDNA was generated from purified RNA using the Applied Biosystems cDNA Synthesis Kit.

## qPCR Analysis of Mitochondrial DNA Copy Number and mRNA Transcripts

## Primers designed for three mitochondrial-encoded gene regions were used to assess mitochondrial DNA (mtDNA) copy number in genomic DNA and primers designed for both mitochondrial- and nuclear-encoded mitochondrial genes were used to assess mRNA transcript levels in cDNA extracted from the quadriceps muscle (see above). The primer sequences used are included in the table below. Briefly, genomic DNA or cDNA from each sample extraction was added to the appropriated working qPCR master mix (containing SYBR Green and the relevant primers). PCR conditions included an activation cycle of 95 ⁰C for 10 min followed by 45 amplification cycles of 15 s at 95 ⁰C, 15 s at 60 ⁰C, and 10 s at 73 ⁰C. Cp values were quantified using Light Cycler 480 software. Values were calculated using the ∆Ct method, with data for mtDNA copy number being normalized to values obtained for a nuclear-encoded genomic locus (*Tsc2*) and mRNA levels being normalized to *Rpl13a*.

**Region Forward primer Reverse primer**

d-Loop GGC CCA TTA AAC TTG GGG GT TTC TTC ACC GTA GGT GCG TC

*Mt-Nd1* CGT CCC CAT TCT AAT CGC CA ATG GCG TCT GCA AAT GGT TG

*Mt-Cytb* CTT CAT GTC GGA CGA GGC TT CCT CAT GGA AGG ACG TAG CC

*Tsc2* AAG AAG CCT CTT CTG CTA CC CAG CTC CGA CCA TGA AGT G

**Gene Forward primer Reverse primer**

*Mt-Nd4* TAA TCG CAC ATG GCC TCA CA GCT GTG GAT CCG TTC GTA GT

*Mt-Cytb* CTT CAT GTC GGA CGA GGC TT CCT CAT GGA AGG ACG TAG CC

*Sdha* TCT TCG CTG GTG TGG ATG TC CTT CAG CAC CTG TCC CTT GT

*Mt-Co2* AAC CGA GTC GTT CTG CCA AT CTA GGG AGG GGA CTG CTC AT

*Rpl13a*  GGA GTC CGT TGG TCT TGA GG GGC CAA GAT GCA CTA TCG GA

## Preparation of protein lysates and western blotting

Skeletal muscle homogenates were prepared from ~30-50 mg of frozen quadriceps in RIPA buffer using stainless steel beads and a Qiagen tissue lyser. Homogenates were centrifuged at 4⁰C for 10 min at 14,000G, after which the protein concentration of supernatants was determined by Bradford assay. Lysates of equal protein concentration were prepared in 2x Laemli buffer containing 2-mercaptoethanol and heated at 37⁰C for 5 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for western blotting. After ponceau staining to ensure equal protein loading, membranes were blocked in BSA for 1 hr and incubated overnight in total OXPHOS rodent WB antibody cocktail (Abcam) at 4⁰C. Blots were visualized after a 1 hr incubation with infrared anti-mouse secondary antibody, using a LI-COR Odyssey fluorescent western blotting system. Protein expression was quantified using densiometry (Image Studio Lite, LI-COR).

## Citrate synthase activity

Muscle homogenates were prepared in KCl-EDTA buffer (pH 7.4) from ~10-40 mg of frozen quadriceps. Following 3 freeze-thaw cycles, samples were centrifuged at 4⁰C for 10 min at 1000 G to settle cellular debris. Supernatants were analyzed for citrate synthase activity using a modified method of (14). Briefly, aliquots of supernatant were added to the appropriate wells of a 96 well microplate containing an assay solution comprised of 72.5 mM Tris, 0.45 mM acetyl CoA and 0.1 mM 5,5’-dithiobis-2-nitrobenzoate (DTNB) (pH 8.3). After monitoring the plate for possible background activity, activity reactions were initiated by the addition of 0.5 mM oxaloacetic acid to each well. Changes in absorbance at 405 nm were recorded for each well every 9-11 sec over 3 min at room temp. Citrate synthase activity was calculated using the extinction coefficient for DTNB (which is reduced by the CoA-SH released during the cleavage of acetyl-CoA by citrate synthase).

## Statistics

Statistics and calculations were performed using Microsoft Excel and R version 3.1.1 (15). For longitudinal data, mixed linear models were used and χ2 tests were performed to determine the significance of the MCP230 treatment. Mixed linear models used the R package lme4 (version 1.1-7 (16)). In all cases, normality of the data and models were determined via Shapiro-Wilk Test and equal variance was tested using Levene’s test from the car package (version 2.0-21 (17)). In cases where cabosil and saline treatment were not significantly different, these data were combined and designated as “Control”. For energy expenditure calculations, we performed an ANCOVA analysis with lean body mass and the treatment group as non-interacting covariates and the averaged light or dark VO2 as the responding variable as described in (18). Statistical significance was designated as a p-value <0.05.

# Results

## Gestational exposure to MCP230 leads to increased weight gain in pups

To test whether gestational exposure to an environmentally persistent free radical (EPFR), we treated pregnant females with two exposures of MCP230 on day 10 and 17 of gestation. As controls, mice were either exposed to cabosil (the non-conjugated particulate without the EPFR group) or saline. These mice were then birthed, left with their dams and weaned at 21 days of age. At 10 weeks of age, mice were placed on a high fat diet consisting of 45% of calories from fat (see Figure 1A).

As shown in Figure 1B, mice that were pre-treated with MCP230 had a higher body weight and proceeded to gain more weight during the diet. At the end of the 12-week diet, we observed a XXg (XX%) increase in body size (Figure 1B). We assessed the body composition and observed significant elevations in both fat mass (10.6% increase, p=0.011) and fat-free mass (10.8% increase, p=2.2x10-4) in these mice (Figures 1C and D). The relative adiposity of these mice as determined by the percent fat mass was not different between groups (Figure 1E).

We next evaluated the extent of obesity related co-morbidities in these mice. We observed no changes in fasting blood glucose as a result of MCP230 exposure (Figure 2B). As shown in Figure 2C, we also did not observe any changes in serum insulin concentrations, suggesting that there was similar insulin sensitivity between the saline and MCP230 treated mice after high fat diet.

To test whether there were any other changes in key metabolic hormone levels, we evaluated the levels of a panel of hormones in fed- and fasted-state serum. As shown in Figures 2E-F, we did not observe any changes in resistin or PAI-1 levels. Leptin was modestly elevated in both the fasted and fed conditions (p-value for the effect of MCP230 treatment via ANOVA was 0.011, post-hoc *t-*test p-values were 0.058 for fasted and p=0.097 for fed leptin levels). These elevations in circulating leptin levels are consistent with the increases in fat mass described in Figure 1E. We observed significant elevations in both the fasting and fed state for Ghrelin, GLP-1, glucagon and GIP-1, though the latter did not attain statistical significance (p=0.069 for fasted GIP-1 levels by Wilcoxon Rank Sum Test).

## MCP230-Exposed Mice Have Reduced Caloric Intake

To determine how energy balance was affected in these mice we first examined their food intake, longitudinally throughout the study. As shown in Figure 3A, all mice tended to eat less food each week, though this did not reach statistical significance. Cumulatively, the MCP230 ate less food throughout the diet (-6.3 +/- 1.8 kcal/week/mouse, χ2=11.6, p=8.0 x 10-4, Figure 3B). Through the 12 week high fat diet treatment this corresponds to a 20% reduction in total caloric intake. To determine whether this reduction in food intake occurred at baseline, or only was due to the HFD, we also examined mice, individually housed at 10 weeks of age. These mice also consumed less food. Together, these data suggest that the increased body mass of MCP230 exposed mice was not due to increased caloric intake, as this was substantially less compared to the control mice.

## MCP230 Mice Have Reduced Energy Expenditure

Since the MCP230 mice did not appear to be larger due to excessive caloric intake, we next examined their energy utilization. To evaluate energy expenditure, we placed mice at 9 weeks of age (prior to HFD) in individual cages for indirect calorimetry, physical activity monitoring and evaluation of gas exchange rates.

As shown in Figure 3C, the MCP230 treated mice had lower energy expenditure, as determined by oxygen consumption (VO2). Figure 3D depicts the energy expenditure, as determined by the VO2 for each mouse, plotted against the animal’s fat-free mass. Accounting for change in lean mass is necessary due to known associations between this covariate and oxygen consumption rates (18). Based on these calculations, we observed decreased energy expenditure of the MCP230 treated mice in both the dark (-19.1%, p=0.020) and light (-16.8%, p=0.031) phases.

To determine whether these decreases in energy expenditure were correlated with changes in locomotor activity, we simultaneously monitored ambulatory activity of these mice. As shown in Figure 3E, we observed a 21.4% reduction in physical activity in the dark phase (p=0.040) and a 26.2% decrease in light phase locomotor activity (p=0.0099) for the mice exposed to MCP230, compared to the control mice.

We next evaluated energy substrate preference by analyzing the respiratory exchange ratio of the three groups. When this ratio nears 1, that indicates preference of carbohydrates as fuel, and as it nears 0.7 it indicates utilization of mainly lipids (19). Although there was no difference between MCP230 and cabosil treated mice, we did observe a significant elevation (carbohydrate preference) of the saline treated mice, relative to either the control (cabosil) or EPFR (MCP230) treated mice in both the light and dark phases (Figure 3F). These data indicate that the unconjugated particle exposure itself (though not the EPFR group) may alter substrate preference in these mice.

## Skeletal Muscle from MCP230 Treated Mice Have Reduced Mitochondrial DNA Copy Number and a Lower Citrate Synthase Activity

Due to the observed reductions in whole-body oxygen consumption and total energy expenditure, we next explored the hypothesis that there may be defects in the skeletal muscle mitochondria of MCP230-exposed mice. To test this, we first determined mtDNA copy number in quadriceps muscle after the 12 week high fat diet phase. Figure 4A demonstrates that MCP230-exposed mice have a marked reduction in mtDNA copy number relative to the saline-exposed mice, as determined using primers designed for three distinct mtDNA-encoded gene regions. Decreases of 61.2%, 68.0% and 51.9% were observed for the dLoop, *mt-Cytb* and *mt-Nd1*, respectively (p=0.039, p=0.031 and p=0.032, respectively). Citrate synthase activity is strongly associated with skeletal muscle mitochondrial content (20) and it’s activity is rate-limiting for the tricarboxylic acid cycle. Given that mtDNA copy number was reduced with MCP203 exposure, we sought to determine if there were any mitochondrial functional defects in the skeletal muscle from particulate-exposed mice. As shown in Figure 4B, maximal citrate synthase activity was reduced 24.1% in the quadriceps from MCP230-exposed mice (p=0.03). Taken together, these data suggest that mice exposed to MCP230 are likely to have reduced mitochondrial oxidative enzyme content and, as a result, reduced skeletal muscle oxidative capacity, which, along with the reduction in physical activity, would likely contribute to the reduced VO2 seen in these mice.

Indeed, mRNA transcript levels for the mitochondrial- and nuclear-encoded electron transport genes *mt-Nd4* (25.2%), *Sdha* (35.9%), *mt-Cytb* (35.4%) and *mt-Co2* (35.1%) were all also reduced in the quadriceps from MCP230-exposed mice, although not all of these reductions attained statistical significance (p=0.12, p=0.08, p=0.04 and p=0.10, respectively (Figure 5). To determine whether similar differences in skeletal muscle mitochondrial electron transport enzymes were also present at the protein level, we next measured the relative expression of several electron transport chain proteins via western blotting (Figure 4C). Interestingly, we observed an increase in the relative abundance of the two of the five proteins measured in skeletal muscle from mice treated with MPC230 compared to the saline-treated mice; NDUFB8 and ATP5A were elevated 66.2% (p=0.042) and 64.2% (p=0.022), respectively, whereas the other three electron transport proteins measured were expressed similarly between the two groups. This finding suggests that functional impairments in the oxidative capacity of the skeletal muscle are likely to be secondary to the ability of the myocyte to generate mitochondrial enzymes at the protein level.

# Discussion

In this study we have tested the metabolic effects of a limited gestational exposure to an environmentally persistent free radical associated with particulate matter as a mimic of combustion derived pollutants. We have noted that these mice grew larger, despite reductions in food intake, and that this is potentially due to reduced energy expenditure and impaired mitochondrial oxidative function in skeletal muscle.

One potential explanation for the reductions in energy expenditure and mitochondrial number is the observed reduction in physical activity for MCP230-exposed mice. It is also possible that muscle weakness, due to reduced skeletal muscle oxidative capacity, could contribute to the reduced physical activity of MCP230-exposed animals. Both of these hypotheses are consistent with cross-sectional studies showing negative associations between pollutant exposure and leisure time physical activity (21) and exercise performance (22–24). Our current data are unable to determine whether reduced mitochondrial function is the primary cause of these reductions in energy expenditure or if this observation is secondary to a reduced propensity for physical activity. However, our observations of reductions in mitochondrial DNA, citrate synthase activity and mRNA transcripts support the possibility that gestational treatment with EPFRs may exert their effects on energy expenditure by directly affecting skeletal muscle mitochondrial oxidative function.

The mechanisms by which gestational EPFR treatment may result in reduced mitochondrial function are not yet clear. These data are consistent with chronic models of PM2.5 treatment, which show reduced mitochondrial numbers in white adipose tissue (7, 8). Analyses of placental tissues from mothers showed a strong correlation between late-gestational PM10exposure and placental mitochondrial DNA content (25). Given the elevated sensitivity of mitochondria to free radicals and oxidative stress, it is reasonable to hypothesize that during development, EPFR-mediated mitochondrial damage may result in chronic decreases in mitochondrial oxidative function, either directly, via reactive oxygen species, or indirectly, via inflammatory processes. Based on our current protocol, mice are treated with EPFRs after inheritance of maternal mitochondria, indicating that this mitochondrial damage occurs *in situ* in the progeny. In contrast to previous studies that use chronic pollution models (1, 4, 3, 6, 26), we did not observe any differences in insulin sensitivity (via glucose/insulin levels), indicating that the effects of gestational particulate exposure do not mimic the effects of chronic exposure, and the risk profiles and mechanisms associated with these exposures may differ.

Another potential contributor to the reduced energy expenditure we observed in MCP230-exposed mice is ghrelin. Ghrelin is an orexogenic peptide hormone that exerts its actions primarily through hypothalamic signaling (27). Ghrelin concentrations are typically elevated with fasting (See Figure 2G; (28, 29)), acting to increase hunger signals and down-regulate energy expenditure pathways (27, 30). Thus, although the elevated ghrelin levels observed in the MCP230-exposed mice could be a result of these mice eating less (Figure 2A and B), the finding that MCP230-exposed mice have higher ghrelin levels could also help explain their reduced VO2 and physical activity. In the context of reduced observed food intake, it is likely that the elevated ghrelin levels in the MCP230 are a response to their reduced food intake. This potential counter-regulatory mechanism is consistent with observations that ghrelin levels are reduced with obesity (31–33). In contrast, elevations in GLP-1 can inhibit food intake (34, 35). Therefore, there are likely to be many other factors contributing to the reduced appetite and lower energy expenditure of the MCP230-exposed mice.

In conclusion, we have investigated the effects of limited, gestational exposure to combustion-derived pollutants in a mouse model of diet-induced obesity. Our findings show that even brief gestational exposure to environmental pollutants such as EPFR’s can result in chronic changes in growth, metabolism and energy balance. These changes are associated with skeletal muscle mitochondrial deficits and reductions in physical activity, which likely contribute to reduced oxygen consumption and a predisposition to elevated body weight on a high fat diet. While the mechanisms behind these changes remain to be determined, understanding the impact EPFR’s and other environmental pollutants have on mammalian energy metabolism will allow us to consider regulating air pollution and other sources of EPFR’s.

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# Figure Legends

**Figure 1: Gestational treatment of MCP230 leads to mice with elevated weight gain on high fat diet.** A) Schematic of treatment. B) Body weight throughout the HFD treatment. C-E) After 12 weeks of HFD, body composition was determined in the fed state (ZT12). Asterisks indicate p<0.05 via a Student’s *t*-test (C-D).

**Figure 2: Gestationally MCP230 treated mice have no changes in liver triglycerides or insulin sensitivity but have elevations in ghrelin.** A) Liver triglycerides and B) blood glucose levels were determined from mice after a 16h fast at approximately ZT4. C-J) Fed (ZT12) and fasted (ZT4) serum hormone levels were determined. Asterisk indicates p<0.05 via a Wilcoxon Rank Sum Test.

**Figure 3: Decreased food intake and energy expenditure in pups from gestationally treated MCP230 mice.** Food intake per mouse was calculated on a A) weekly and B) cumulative basis throughout the High-Fat Diet Treatment. C) Oxygen consumption rates for mice in metabolic cages. Shaded area indicates the dark phase. D) Analysis of oxygen consumption, normalized to lean body mass in light and dark phase. Each dot represents the average oxygen consumption per mouse. E) Quantification of x-phase ambulatory movement during the light and dark phases. F) Respiratory exchange ratio of each group. In this case, the saline and cabosil groups were not combined due to a significant depression of the ratio in both the cabosil and MCP230 treated groups. Asterisks indicate p<0.05 by Mixed Linear Models Compared by χ2 test (B), ANOVA (D), Student’s *t*-test (E) or Wilcoxon-Rank Sum Test (F).