Exercise prevents maternal high-fat diet-induced hypermethylation of the Pgc-1α gene and age-dependent metabolic dysfunction in the offspring

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Abnormal conditions during early development adversely impact later health. We investigated whether maternal exercise could protect the offspring from adverse effects of maternal HFD with a focus on the metabolic outcomes and epigenetic regulation of the metabolic master regulator, peroxisome proliferator activated receptor \( \gamma \) co-activator-1 \( \alpha \) (Pgc-1 \( \alpha \)). Female C57BL/6 mice were exposed to normal chow, HFD, or HFD with voluntary wheel exercise for 6 weeks prior to and throughout pregnancy. Methylation of the Pgc-1 \( \alpha \) promoter at CpG site -260 and Pgc-1 \( \alpha \) mRNA expression were assessed in skeletal muscle from neonatal and 12 month-old offspring, and glucose and insulin tolerance tests (GTT and ITT, respectively) were performed in the female offspring at 6, 9 and 12 months. Hypermethylation of the Pgc-1 \( \alpha \) promoter caused by maternal HFD was detected at birth, which was maintained to 12 month of age with a trend of reduced Pgc-1 \( \alpha \) mRNA (\( P = 0.065 \)) and its target genes. Maternal exercise prevented maternal HFD-induced Pgc-1 \( \alpha \) hypermethylation and enhanced Pgc-1 \( \alpha \) and its target gene expression concurrent with amelioration of age-associated metabolic dysfunction at 9 months of age in the offspring. Therefore, maternal exercise is a powerful lifestyle intervention in preventing maternal HFD-induced epigenetic and metabolic dysregulation in the offspring.
INTRODUCTION

The prevalence of maternal obesity is increasing at an alarming rate. Even more disturbing is that maternal obesity increases susceptibility of the offspring to developing metabolic disease later in life and therefore contributes to a vicious cycle of transgenerational transmission of disease (1,2). Encouragingly, accumulating evidence has shown that maternal exercise has beneficial effects on offspring metabolic outcomes (3–8). These benefits include improved glucose tolerance with increased glucose clearance in skeletal muscle and adipose tissue (3). However, it is unknown whether introduction of maternal exercise can protect offspring from maternal high-fat diet (HFD)-induced metabolic dysfunction and what the underlying mechanism(s) of this developmental programming might be.

A promising candidate for parent-offspring transmission of metabolic dysfunction is the epigenetic modification of metabolically important genes, through DNA methylation, histone modifications or microRNA regulation (5–10). DNA methylation typically occurs in differentiated cells at cytosine of CpG dinucleotide pairs. Methylation of the promoter region can block transcription and silence gene expression (13–15). Peroxisome proliferator activated receptor γ co-activator-1 α (PGC-1 α), a transcriptional co-activator, is a master gene of mitochondrial biogenesis and oxidative metabolism (16,17). It has been shown that PGC-1 α promoter is hypermethylated, which negatively correlates with mRNA expression in skeletal muscle of type 2 diabetes patients (18). Furthermore, hypermethylation of CpG site -260 is sufficient to reduce PGC-1 α promoter activity (18). Thus, methylation of the PGC-1 α promoter in skeletal muscle is an epigenetic modification with important consequences relevant to the development of metabolic disorders.
Here, we employed epigenetic analysis in well-established animal models of diet-induced obesity and voluntary wheel running to test the hypothesis in mice that maternal HFD-induced age-dependent metabolic dysfunction in the offspring is linked to Pgc-1α promoter hypermethylation and reduced Pgc-1α mRNA expression and function. More importantly, we also tested whether maternal exercise would mitigate the metabolic and epigenetic abnormalities.
MATERIALS AND METHODS

Animals. Female mice (C57BL/6, 8 weeks old, n = 4/group) were subjected to the following diet-activity interventions for 6 weeks prior to and throughout pregnancy: normal chow diet with sedentary activity (Sed-NC), 60% HFD (Research Diets, NJ) with sedentary activity (Sed-HF) or HFD with exercise training (voluntary running; Ex-HF). Mice were housed individually in cages equipped with running wheels, which were locked for the sedentary groups. At the time of mating a sedentary male mouse (C57BL/6, 14 weeks old, n = 6 total), on normal chow diet, was placed in the cages overnight and pregnancy was confirmed by vaginal plug. The female mice continued on the same diet-activity intervention until term. All dams and offspring were fed normal chow with sedentary activity during lactation and after weaning (21 days). Glucose and insulin tolerance tests (GTT and ITT) were performed for the female offspring (Sed-NC = 8, Sed-HF = 4, Ex-HF = 5) at 6, 9 and 12 months of age by measuring tail vein blood glucose following glucose (3.0 g/kg body weight, i.p.) and insulin (1 U/kg body weight, i.p.) injection, respectively (19). At 12 months, DEXA was performed for body composition (20). All hind limb muscles were harvested, after the mice were humanely euthanized, from two pups per litter at birth and the quadriceps muscle from the remaining littermates at 12 months of age. All procedures were approved by the Animal Care and Use Committee of University of Virginia.

DNA Methylation Analysis. Genomic DNA was isolated and bisulfite converted using methylCode bisulfite conversion kit (Invitrogen Life Technologies, Carlsbad, CA). PCR primers spanning the CpG site at -260 of the Pgc-1α promoter were designed using the PyroMark primer design software (Qiagen, Valencia, CA). PCR was performed using the PyroMark PCR kit (Qiagen) with forward TGAGTTATATGTGAGTGGGGTTT and reverse CCAACCTCCCTTCTCTATAC primers and
the following conditions; 1 cycle at 95°C for 15 min, 50 cycles at 94°C for 30 sec followed by 54°C for 30 sec and then 72°C for 30 sec, and final extension at 72°C for 10 min. The PCR product (3 μl) was resolved by electrophoresis on 2% agarose gel to confirm identity of the product. Sequencing with the primer TGAGTTATTATGTGAGTA was performed using the PyroMark Q24 pyrosequencing machine (Qiagen). Non-CpG cytosines acted as internal controls for bisulfite conversion efficiency since they are not methylated and expected to have 100% conversion to uracil and upon amplification identified as thymine. The data is reported as percent methylation by determining the number of times the site exists as cytosine in the context of total number of times the site is detected as thymine or cytosine. Data was analyzed using PyroMark Q24 software (Qiagen).

**mRNA Analysis.** PCR of total RNA was performed as previously described (21) using primers and probe for \( Pgc-1 \alpha \) (Mm00470540_m1), Glucose transporter 4 (\( Glut4 \); Mm00436615_m1), Cytochrome c oxidase subunit 4 (\( Cox4 \); Mm00446387_m1), Cytochrome c (\( Cyt c \); Mm00470540), Vascular endothelial growth factor a (\( Vegfa \); Mm00437306_m1), Myosin heavy chain 2a (\( Myh2a \); Mm01332564_m1), Superoxide dismutase 1 (\( Sod1 \); Mm01344233_g1) and hypoxanthine guanine phosphoribosyl transferase 1 (\( Hrpt1 \); Mm00446968_m1) (Applied Biosystems, Foster City, CA). **mRNA expression** was normalized by \( Hrpt1 \).

**Statistic analysis.** Data are presented as mean ± SE. Comparisons were done by one-way ANOVA followed by the Student Newman-Kuels post hoc test with \( P < 0.05 \) as statistically significant. For GTT and ITT analyses, two-way ANOVA with repeated measures was conducted, and if an interaction was observed, one-way ANOVA was performed for each of the time points among different groups.
RESULTS

Maternal HFD induces muscle-specific hypermethylation of the Pgc-1α promoter in the offspring at birth, which is attenuated by maternal exercise--To investigate the epigenetic impact of maternal diet and exercise on the offspring, we assessed Pgc-1α promoter methylation at CpG site -260 (Fig. 1A) in muscle and liver from the offspring at birth. The Pgc-1α promoter was hypermethylated ($P < 0.05$) in skeletal muscle of Sed-HF offspring compared with Sed-NC offspring (Fig. 1B), which was attenuated in Ex-HF offspring (Fig. 1B). No differences in methylation levels were observed in the liver (Fig. 1C). Pgc-1α mRNA levels were similar amongst the groups (Fig. 1D), and there was no correlation between methylation and mRNA expression (Fig. 1E). These findings indicate that maternal diet and exercise imposes muscle-specific epigenetic modification of Pgc-1α in the offspring.

Maternal exercise prevents maternal HFD-induced Pgc-1α hypermethylation and reduction of Pgc-1α mRNA in adult offspring--To determine whether maternal HFD-induced hypermethylation of the Pgc-1α promoter in offspring muscle was sustained to adulthood, we assessed Pgc-1α methylation in the 12-month old littermates. Sed-HF offspring displayed hypermethylation of the Pgc-1α promoter ($P < 0.05$) compared with Sed-NC offspring (Fig. 2A), which was completely prevented in Ex-HF offspring (Fig. 2A). There was a trend ($P = 0.056$) for Pgc-1α methylation to negatively correlate ($\rho = -0.48$) with its mRNA levels (Fig. 2C). Pgc-1α mRNA in muscle of Ex-HF offspring was significantly higher ($P < 0.05$) than both Sed-NC and Sed-HF offspring (Fig. 2B), and there was a trend ($P = 0.065$) for reduced Pgc-1α mRNA (~50%) in Sed-HF offspring compared with Sed-NC (Fig. 2B). Glut4, Cox4 and Cyt c mRNA, but not Myh2a and Sod1 mRNA, exhibited a similar expression pattern to that of Pgc-1α, such that expression was significantly higher in Ex-HF offspring.
skeletal muscle at 12 months of age (Fig. 2D). In addition, Cox4 and Cyt c mRNAs were lower ($P < 0.05$) in Sed-HF offspring, with a similar trend ($P = 0.072$) observe for Glut4 mRNA (Fig. 2D). There were no significant differences in Myh2a and Sod1 mRNA expression amongst the groups (Fig. 2D). Postnatal growth, body weights, fat and lean body mass were similar between groups (Fig. 2E-G).

*Maternal exercise protects offspring from maternal HFD-induced metabolic dysfunction with aging--* To investigate whether the epigenetic mark on Pgc-1α was associated with metabolic outcomes, we assessed glucose and insulin tolerance in the aging offspring. There were no differences in GTT and ITT analyses between groups at 6 months (Fig. 3A-C). At 9 months, Sed-HF offspring displayed glucose intolerance (Fig. 3D; $p<0.01$ at 30 min and $p<0.05$ at 60 min) with greater AUC (Fig. 3E; $P < 0.01$) compared with Sed-NC offspring. Maternal exercise prevented the maternal HFD-induced metabolic dysfunction at this age (Fig. 3D-F). We did not find statistically significant differences, at 12 months of age (Fig. 3G-I). In a separate cohort, maternal exercise, without HFD as a negative control, had no impact on Pgc-1α methylation in offspring skeletal muscle (Fig. 4A and B) or glucose tolerance at 18 weeks of age (Fig. 4C and D).
DISCUSSION

Our findings demonstrate a link between the maternal condition, epigenetic modifications to the gene of a master metabolic regulator in the offspring and later metabolic health outcomes. We observed that the $Pgc-1\alpha$ promoter was hypermethylated in skeletal muscle, but not in liver, of newborns from dams exposed to HFD. This epigenetic mark was maintained up to 12 months of age and exhibited a negative correlation with $Pgc-1\alpha$ and its target transcript levels. Importantly, these findings were accompanied by an age-dependent glucose intolerance at 9 months. Although a definitive cause and effect cannot be confirmed, our findings strongly support an epigenetic mechanism in the parent-offspring transmission of metabolic disease and suggests maternal exercise as an intervention to halt the vicious cycle with powerful positive epigenetic influences.

We have for the first time shown that maternal HFD induces hypermethylation of the $Pgc-1\alpha$ promoter in offspring skeletal muscle. Importantly, this occurred in a region of the $Pgc-1\alpha$ promoter known to be hypermethylated in patients with type 2 diabetes (18). It is possible that systemic effects of maternal HFD, such as elevated circulating lipids and inflammatory cytokines that can enter the fetal circulation, impair the gestational environment and alter DNA (cytosine-5-)methyltransferase (Dnmt) activity (9). Indeed, $PGC-1\alpha$ promoter methylation has shown to be increased by TNF$\alpha$, palmitate or oleate treatment in primary human myotubes (18). This epigenetic modification is likely a result of altered DNMT3b isoform (18). Regulation of DNMT activity can be influenced by microRNAs, phosphorylation, translational activation and expression, and thus it will be important in future studies to dissect the precise influence of maternal HFD on skeletal muscle DNMT isoforms. Currently, the only findings relevant to this question are altered expression of DNMT isoforms in liver
of offspring from undernourished dams (18, 19), providing a hint to their involvement in developmental programming.

Whether the epigenetic modification has functional consequences is of great significance for disease outcomes. In general, CpG methylation of a promoter region represses the transcription. Although non-CpG methylation of PGC-1α has been associated with metabolic disease (18), the functional relevance is unclear and has yet to be elucidated. In the current study, we focused on methylation of CpG site -260 of the Pgc-1α promoter to ensure that the findings were functionally meaningful. Interestingly, the differences in Pgc-1α promoter methylation at birth in offspring skeletal muscle did not impact mRNA expression. We speculate that rapid proliferation and differentiation of myogenic cells during this critical period of growth requires active transcription of Pgc-1α. In contrast, in fully differentiated adult skeletal muscle where myogenic cells are quiescent, DNA methylation may have more influence on gene transcription. Indeed, we observed that differences in Pgc-1α promoter methylation were associated with changes in gene expression by up to 50% in the adult offspring. Furthermore, downstream target gene Glut4, Cox4 and Cyt c mRNA expression but not Myh2a and Sod1 mRNA, mirrored that of Pgc-1α and provides further evidence for the functional importance of epigenetic regulation of Pgc-1α. Importantly, in skeletal muscle of humans with type 2 diabetes a similar degree of PGC-1α hypermethylation corresponded to reduced mRNA expression by ~35% (18). Together, these data links hypermethylation of the Pgc-1α promoter to its gene expression in adult offspring.

The most exciting finding of this study is that maternal exercise protects offspring from maternal HFD-induced metabolic dysfunction at 9 months of age. Due to low sample size and increased variability within groups that naturally develops with aging, we did not achieve statistical differences in metabolic phenotype between groups at 12 months of age. Regardless, our findings at 9 months of age were in
parallel with prevention of skeletal muscle *Pgc-1α* promoter hypermethylation and preserved *Pgc-1α* mRNA later in life. These benefits appeared to be specific to the condition of maternal HFD as maternal exercise, without HFD as a negative control, had no effect on *Pgc-1α* methylation or glucose tolerance in adult offspring. These findings suggest that maternal exercise suppresses the maternal HFD-induced hypermethylation of *Pgc-1α* in the offspring, rather than initiates an independent process of epigenetic modification, such as demethylation. Since exercise training in mothers fed HFD prevented the body weight increase induced by HFD (not shown) it is likely that the positive impact of exercise is mediated by suppression of dislipidemia and associated systemic inflammation that alter the gestational environment (9). Indeed, exercise training has positive impacts on blood lipid profiles and inflammatory cytokines associated with obesity as reported in adult male mice (24). We, therefore, speculate that reduction in circulating factors that have been previously shown to increase *PGC-1α* promoter methylation (18) is responsible for the maternal-exercise mediated protection to the offspring. Future studies will be required to investigate the maternal HFD-induced factors that influence offspring epigenetic regulators and the physiological changes induced by maternal exercise that are associated with prevention of epigenetic modifications.

In summary, we have provided evidence that maternal HFD-induced metabolic dysfunction in the aging offspring, could be significantly ameliorated by maternal exercise. Methylation of the master metabolic regulator, *Pgc-1α*, at CpG site -260 in the offspring is sensitive to the maternal condition, and the epigenetic mark laid during embryonic development is maintained to adulthood. Hypermethylation of the *Pgc-1α* promoter has a negative impact on gene expression and metabolic outcomes as mice age. Our most novel finding is that exercise intervention protects the fetus from adverse epigenetic modifications induced by maternal HFD, resulting in preserved gene expression and
metabolic function in later life. The findings are critical to stem the cycle of developmental programming of disease and can be readily translatable to human practice with significant implications for public health.
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Author contributions: R.C.L performed the animal experiments, data analysis and interpretation, and wrote the manuscript. T.L, M.O, M.Z and K.H provided technical support, contributed to discussion and reviewed the manuscript. J.J.C and Z.Y conceived the experiments, contributed to discussion and interpretation and wrote the manuscript. The authors have no conflicts of interest. Drs. Rhianna Laker and Zhen Yan are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES


FIGURE LEGENDS

**Figure 1.** Maternal exercise prevents maternal HFD-induced hypermethylation of the Pgc-1α promoter in skeletal muscle in the offspring. Pgc-1α promoter methylation and mRNA expression was assessed by pyrosequencing and real-time PCR, respectively, in offspring skeletal muscle and liver at birth. Schematic presentation of the structural feature of the Pgc-1α promoter (A). Circles represent CpG islands, labeled by the base pair number relative to the transcription start site, with site -260 highlighted in red. Open rectangles represent transcription factor binding sites. An arrow marks the transcription start site. Pgc-1α promoter methylation at CpG site at -260 in muscle (B) and liver (C). Pgc-1α mRNA in offspring skeletal muscle (D) and its correlation with Pgc-1α methylation status (E). * indicates $P < 0.05$.

**Figure 2.** Maternal HFD-induced Pgc-1α hypermethylation is maintained with reduced gene expression and abnormal metabolic function in aging mice. Pgc-1α promoter methylation and mRNA expression was assessed by pyrosequencing and real-time PCR, respectively, in offspring skeletal muscle at 12 months of age. Pgc-1α promoter methylation at CpG site at -260 (A), Pgc-1α mRNA expression (B), correlation between Pgc-1α methylation and gene expression (C) and mRNA expression of Glut4, Cox4, Cyt c, Myhc2a and Sod1 (D) in skeletal muscle at 12 months of age. Body weight and composition are presented as growth profile from birth to 12 months (E), percent lean body mass (F) and fat mass (G) measured by DEXA at 12 months of age in female offspring. * and ** indicate $P < 0.05$ and 0.01, respectively.

**Figure 3.** Maternal exercise protects offspring from maternal HFD-induced metabolic dysfunction. Whole body glucose tolerance and insulin sensitivity was assessed in aging female
offspring following a bolus i.p. injection of glucose or insulin, respectively by measuring blood
glucose over time. Blood glucose during GTT and AUC at 6 (A and B, respectively), 9 (D and E,
respectively) and 12 (G and H, respectively) months of age. Blood glucose during ITT at 6 (C), 9 (F)
and 12 (I) months of age. * and ** indicate $P < 0.05$ and 0.01, respectively.

**Fig 4. Maternal exercise alone does not affect $PGC-1\alpha$ promoter methylation in the skeletal
muscle or glucose tolerance in the offspring.** $Pgc-1\alpha$ promoter methylation was assessed by
pyrosequencing in skeletal muscle, and glucose tolerance was assessed following a bolus i.p. injection
of glucose by measuring blood glucose over time, in 18 week old female and male offspring. $PGC-1\alpha$
promoter methylation at CpG site -260 females and males (A and B, respectively) and blood glucose
during GTT in females and males (C and D, respectively).
A

CpG sites

NFAT

CRE

USF

Sp1

MyoD

Pgc-1a gene

B

% Methylation in skeletal muscle

% Methylation in liver

C

D

Relative gene expression

E

% Methylation

mRNA expression

P = 0.85

\( \rho = 0.04 \)