Maternal low protein diet causes body weight loss in male, neonate Sprague-Dawley rats involving UCP-1 mediated thermogenesis

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Abstract

Brown adipose tissue (BAT) plays an important role in regulating body weight (BW) by modifying thermogenesis. Maternal low protein (LP) diets reduce offspring birth weight. Increased BAT thermogenesis *in utero* may be one mechanism for the lower BW. However, whether maternal LP nutrition alters BAT thermogenesis and BW of offspring *in-utero* is not yet known. We fed obese-prone Sprague-Dawley dams 8% low protein (LP) or 20% normal protein (NP) diets for 3 weeks prior to breeding and through pregnancy. BW and gene expression of interscapular BAT (iBAT) thermogenic markers were measured in male fetal (gestation day 18) and neonatal (day 0 or 1) offspring. BW of neonatal LP males was lower than NP males but no difference was observed in females. Gene and protein expression of UCP-1 and transcription factors PRDM16 and PPAR α in iBAT were 2- to 6-fold greater in LP than NP male neonatal offspring. FND5, a precursor of irisin and activator of thermogenesis, was expressed 2-fold greater in neonatal LP iBAT than NP males. However, fetal iBAT UCP-1, PRDM16, PPAR α and irisin mRNA did not differ between LP and NP groups. Maternal LP diet had no effects on placental irisin and UCP-2 expression. These results suggest that prenatal protein restriction increases the risk for low BW through mechanisms affecting full-term offspring iBAT thermogenesis but not greatly altering fetal iBAT or placental thermogenesis.

Introduction

In humans [1] and in animal models [2-6] maternal low protein (LP) diets reduce offspring birth weight. Low birth weight (LBW) promotes rapid adipose tissue growth during adolescence, further adult-onset weight gain [2], and increased risk for insulin resistance [7]. Alterations in secretion of hormones of the growth hormone-insulin-like growth factor axis [8-10] contribute to LBW. However, other mechanisms are also likely to be involved. Dysregulation of energy metabolism could promote LBW. However, the influence of a maternal LP diet on reprogramming *in-utero* function of metabolic tissues such as brown adipose tissue (BAT) has not yet been determined. We have shown that male offspring of dams fed maternal LP diets have low birth weight [7]. Uncoupling protein-1 (UCP1) is a marker of BAT thermogenesis and an important regulator of body temperature, body weight, and insulin resistance [11].

The placenta, another source of *in utero* heat for the neonate [12-14] could influence the required rate of fetal thermogenesis. As such, we tested whether placental and fetal thermogenesis is also regulated by maternal LP diet. For the current studies, we hypothesized that maternal LP diets induce UCP-1 during the fetal developmental period to increase thermogenesis resulting in LBW. We also hypothesized that placental thermogenesis, measured by irisin and UCP-2 expression, would be increased in dams fed a LP diet. Finally, hormones that bind to nuclear receptors, including sex hormones and thyroid hormones, upregulate UCP-1 [13, 15, 16]. Therefore, we hypothesized that estrogen, testosterone, and thyroid hormones induce BAT thermogenesis, which could lead to LBW.

Materials and methods

1.1. Study design and animals.

Obese-prone Sprague-Dawley female rats (Charles River, Wilmington, MA) were fed an AIN93-based diet containing either 8% (low protein, LP) or 20% protein (normal protein, NP) starting 3 weeks prior to breeding. Obese-prone male breeders were maintained on a normal chow diet. Dams were maintained on their respective diets throughout pregnancy. Dams and day 0-1 neonatal pups were sacrificed by CO₂ inhalation. Neonate pup weights were measured in individual weight boats using an analytical balance (Sartorius Inc., Elk Grove, IL) that reads to 3 decimal places. Fetuses and placental tissues were collected at 18-20 days gestation (E18; approximately 2 days prior to 21 days of gestation). Interscapular BAT (iBAT) tissue samples were collected at 18-20 days gestation or day 0-1 after birth. Harvested tissues were either immediately frozen in liquid nitrogen or cultured.

1.2. Real-time RT-PCR.

Total RNA was extracted from brown adipose tissue or placental tissue using a RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) and used to measure peroxisome proliferator-activated receptor alpha (PPAR α), uncoupling protein-1 (UCP-1), UCP-2, UCP-3, PR domain containing 16 (PRDM16), fibronectin type III domain-containing protein 5 (FNDC5), peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1 α), cluster of differentiation 137 (CD137), or deiodinase 2 (Dio2) mRNA expression by real-time RT-PCR method. Primers were purchased from Integrated DNA Technology (IDT, Coralville, IA) (Table 1). The Taqman assay reagents and

endogenous control (18S-rRNA) were purchased from Applied Biosystems (cat # 4319413E-1307061, Foster City, CA) and were used to measure Ct values using the ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA). Normalizations of the target gene expression were done using the 18S-rRNA.

1.3. Western blot analysis.

15 µg of protein from isolated mitochondria was suspended in 2.5 µL of sample buffer and 1µL 1M DTT to run electrophoresis using 10% Bis-Tris gel (Life Technologies). Protein was transferred to PVDF membrane, washed, and hybridized with primary rat UCP-1 antibody (abcam, Cambridge, MA), and beta-actin (Santa Cruz Biotechnology, Dallas, TX) at 4°C overnight. The membrane was washed with 1X PBS containing 0.1% Tween and hybridized with a secondary antibody (goat anti-mouse – 1:20,000 and goat anti-rabbit – 1:20,000) for 1 hr in buffer containing 0.1% Tween and 0.01% SDS. Images were developed using an Odyssey Infrared Imager (Li-Cor Biosciences Inc., Lincoln, NE).

1.4. Measurement of catecholamines and sex hormones.

Plasma was sent to Vanderbilt University, Hormone Assay & Analytical Services Core (Nashville, TN) to measure catecholamine levels by HPLC. Brown adipose tissue and plasma was sent to University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA) to measure estrogen, testosterone, and progesterone levels.

1.5. Brown adipose tissue harvesting/Cell Culture.

Brown adipose tissue extracted from pups at 18-21 days gestation was cultured in 10% fetal bovine serum/90% low glucose DMEM with penicillin-streptomycin and stimulated with estrogen, progesterone, or testosterone (Sigma-Aldrich, St. Louis, MO) for 24 hours. mRNA was isolated from brown adipose tissue and gene expression levels of UCP-1, UCP-3, PPAR α , PRDM16, and FNDC5 were measured by qPCR and normalized to 18S rRNA.

1.6. Statistical analysis

Data are reported as fold change of the means $\pm$ standard error of the mean (SEM) or error propagation of SEM. GraphPad PRISM5 software was used for unpaired two-tailed t-tests. However, nonparametric Mann-Whitney two-tailed t-tests were used if variances were significantly different among groups. Linear regression was used to determine strength of the associations between FNDC5 mRNA expression and expression of thermogenic factors and associations between Dio2 and UCP-1 mRNA expression. Statistical significance was set at $P < 0.05$.

2. Results

2.1. Effects of maternal LP diet on neonatal offspring birth weight.

Compared to a prenatal NP diet, a prenatal LP diet led to lower birth weights in neonatal male offspring (Fig. 1, $p < 0.02$). The average weight of the LP diet males was 4.96g versus 5.55g in the NP diet males. There was no difference in female offspring from

145 prenatal NP diet and prenatal LP diet groups. Thus, the rest of the paper focuses on
146 male offspring.

148 **2.2. Maternal LP diet increases UCP-1, PPAR- α , PRDM16, UCP-3, and FNDC5** 149 **mRNA expression in iBAT from neonatal male offspring.**

150 As shown in Fig. 2A and 2B, a maternal LP diet increased ($p < 0.02$) thermogenic UCP-1
151 and UCP-3 gene expression in iBAT of neonatal male offspring in the LP compared to
152 NP group. mRNA levels of other transcription factors linked to UCP-1 transcription
153 activation including PPAR α (Fig. 2C, $p < 0.005$) and PRDM16 (Fig. 2D, $p < 0.05$) were
154 also increased in LP male group while PGC-1 α was not different (Fig. 2E). FNDC5
155 mRNA was increased in the maternal LP diet male offspring iBAT compared to maternal
156 NP diet male offspring iBAT (Fig. 2F), $p < 0.002$). Similarly with UCP-1 mRNA, maternal
157 LP diet increased offspring iBAT UCP-1 protein expression ($p < 0.05$, Fig. 3). FNDC5
158 gene and thermogenic gene transcription factors were positively correlated in both LP
159 and NP groups (Fig. 4A-4C). Additionally, UCP1 gene expression was positively
160 correlated to Dio2 gene expression in both LP and NP groups (Fig. 4D).

162 **2.3. Effects of prenatal LP diet on catecholamines, sex hormone, and thyroid** 163 **concentrations.**

164 Plasma concentrations of catecholamines, estrogen, testosterone, and progesterone of
165 neonatal male rats from LP and NP groups were not different (data not shown).
166 Similarly, brown adipose tissue concentrations of estrogen, testosterone, and
167 progesterone of neonatal male rats from LP and NP groups were not different (data not

shown). Likewise, plasma concentrations of T4 and T3 did not differ between the NP and LP groups (data not shown)

2.4. Effects of prenatal LP diet on thermogenic gene and transcription factor mRNA expression in iBAT from fetal male offspring.

UCP-1 ($p=0.66$), PPAR α ($p=0.49$), FNDC5 ($p=0.13$), PRMD16 ($p=0.30$) and PGC1 α mRNA ($p=0.78$) mRNA levels in fetal male iBAT of LP were not different than the NP fetal males (Fig. 5A-5E).

2.5. Placental tissue expresses PRDM16, CD137, and FNDC5.

Placental tissue connected to male fetuses from dams on a LP or NP diet was collected on E18 and thermogenic markers were measured by qPCR. Placental tissue expression of UCP-1 mRNA was not detected in LP or NP groups (data not shown) while UCP-2 mRNA levels were present, but not different between the two diet groups (Fig. 6D). Gene expression of PRDM16, CD137 (beige adipocyte marker, [17, 18]), or FNDC5 between LP and NP diet placental tissue were not different between LP and NP group placental tissues (Fig. 6A-C).

3. Discussion

We demonstrated a sexually dimorphic response to maternal diet in that neonatal male, but not female offspring, had lower BW when dams were fed a LP diet. In contrast, others have shown that female offspring have lower BW in the LP than NP

group [19, 20]. One possible reason for this difference between studies includes using different strains of rat [19, 20].

Using the male offspring, we demonstrated that neonatal, but not fetal, iBAT mRNA expression of thermogenic genes and transcription markers including UCP-1, UPC-3, PPAR α , and PRDM16 were increased in male offspring of the maternal LP compared to maternal NP group. We hypothesized that differences in sex hormone concentrations could induce iBAT thermogenesis, which could explain LP versus NP differences in iBAT thermogenic responses. Interestingly, neonatal male rats express estrogen and androgen receptors in cultured primary brown adipocytes [21] and sex hormones increase thermogenesis [15]. However, there were no differences in plasma estrogen and testosterone concentrations between neonatal LP versus NP groups (data not shown). Additionally, we treated neonatal male iBAT samples with E2, testosterone, and progesterone and found no increase UCP-1 gene expression or intracellular signaling pathways in the LP or NP samples (data not shown). Moreover, we tested plasma catecholamine levels and found no difference between LP and NP groups (data not shown). These data suggest that increased iBAT UCP-1 in the LP diet is not mediated by sex hormones or catecholamines.

Adult male offspring from dams fed an 8% protein restricted diet have increased serum triiodothyronine 4 (T4) and T3 concentrations [22] and this elevation in T3 led to an increase in UCP levels in brown adipocytes. In cases where rats have been thyroidectomized, UCP-1 levels are decreased 3-fold in brown adipocytes and the rats are hypothermic. T3 replacement therapy alone does not correct the low levels of UCP or the hypothermia. However, T4 replacement therapy rapidly restores both UCP

expression and normothermia. Therefore, intracellular conversion of T4 to more metabolically active T3 by 5'-deiodinase is likely necessary for optimal thermogenic function of iBAT [13]. The levels of D1 and D2 deiodinases are altered in adult rats when their dams were fed a protein restricted diet (8%) during lactation. These adult offspring had increased muscle and liver D1 and decreased muscle D2 activities, no change in thyroid deiodinases, and increased pituitary D2 activity in comparison to offspring from dams that were on a normal protein diet (28%) [23, 24]. The greater D1 in the liver and muscle were considered to contribute to the enhanced circulating T3 observed in the adult offspring from the protein restricted group [24]. Additionally, rats treated with T3 have increased UCP-1 at 22°C in iBAT while at 4°C, only T4 treatment was able to increase UCP-1 levels. Based on these data, we investigated whether prenatal NP and LP diets differentially altered the deiodinase-2 (Dio2) mRNA expression. There was a positive correlation between Dio2 and UCP-1 mRNA expression in male, neonatal iBAT, (Fig. 4D), but the overall expression of neonatal male offspring's Dio2 and plasma concentrations of T4 and T3 were not different between the NP and LP groups (data not shown).

Hormonal changes occur at birth to activate thermogenesis to maintain body temperature when exposed to colder extrauterine conditions [12-14]. To distinguish birth-induced activation of thermogenesis from maternal diet induced programming of thermogenic pathways involving UCP-1 gene expression regulation, we tested UCP-1 expression in both fetal and neonatal iBAT. As shown in Fig. 5, fetal UCP-1, PPAR α , PGC-1 α , PRDM16 were not different between LP and NP groups whereas neonate UCP-1, PPAR α , and PRDM16 were increased in the LP group. Since UCP-1 was not

increased in the fetal LP group it is unclear whether the increased levels that are seen in neonates is due to birth-induced activation of thermogenesis or strictly maternal diet induced programming of the thermogenic pathway. Further studies are needed to measure the expression of non-shivering thermogenesis inhibitors [13] and to determine whether thyroid hormone concentrations are greater in LP neonates thereby increasing UCP-1 expression.

Heat is transferred from placenta to fetus [12-14] and increased thermogenesis requires oxygen consumption by brown adipocytes [25]. Our data do not address whether a maternal LP diet induces placental dysfunction resulting in reduced heat production and thus heat transfer from placenta to fetus. We did, however, determine the mRNA expression levels of thermogenic markers in the placenta connected to male fetuses. Levels of PRDM16, CD137, and FNDC5 in the placenta were not different between the LP and NP groups, suggesting no difference in placental heat production of the LP and NP dams. This is in line with our finding of no differences in expression of UCP-1, PPAR α , PGC-1 α , PRDM16 expression in fetal iBAT. It appears that in utero there is no need for LP male fetuses to generate additional heat via activation of iBAT as the uterine environment provides adequate warmth. However, once the neonate is exposed to a cooler ambient environment after birth, the fetal programming appears to have occurred in the LP male because UCP-1, UCP-3, PPAR α , and PRDM16 mRNA expression rapidly increases in iBAT.

This is the first study showing the presence of FNDC5 in rat placental tissue. Although the functional role irisin plays in placenta is unclear, it is possible that irisin modulates hormones like brain-derived neurotrophic factor (BDNF) [26] which regulates

energy homeostasis in adipose tissue [27, 28]. We also determined that the mRNA levels of UCP-1 were below detectable levels (data not shown). Therefore, we measured the mRNA levels of UCP-2 and found that both LP and NP placenta do express UCP-2, but the expression levels were not different between the two diet groups (data not shown).

Taken together these data demonstrate that a maternal LP diet increases iBAT thermogenic gene expression via activation of PPAR α , PRDM16, and FNDC5 in male rat neonates and that maternal LP diet-induced thermogenesis is not influenced by placental thermogenesis. Future studies are needed to address long term implications of maternal LP diet-induced increases in thermogenesis such as development of metabolic diseases.

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Figure Legends

Figure 1. Effect of maternal low protein diet on birth weight of male and female

offspring. Birth weight (g) of low protein (LP, 8%) and normal protein (NP, 20%) male and female pups. Values are means $\pm$ SEM, n=17-19 for male, n=14-17 for female. Significant * = $p \leq 0.05$ between LP and NP.

Figure 2. Maternal low protein diet increases neonate iBAT thermogenic gene and

FNDC5 mRNA expression. Male iBAT was isolated from day 0-1 neonates and mRNA

expression of (A) UCP1, (B) UCP3, (C) PPAR α , (D) PRDM16, (E) PGC1 α , and (F)

FNDC5 genes were measured by qPCR and normalized to 18S rRNA. Values are fold change $\pm$ SEM, n=7-9 for all markers except for PRDM16 which had samples number ranges of 17-29. Significant * = $p \leq 0.05$ between LP and NP.

Figure 3. Maternal low protein diet increases neonate iBAT UCP-1 protein

expression. iBAT UCP-1 protein expression from day 1 male pups exposed to prenatal

LP or NP diets. Values are means $\pm$ S.E.M.; n= 3-5 per group. Significant * = $p \leq 0.05$

between LP and NP.

Figure 4. Correlation of FNDC5 to UCP1, PPAR α , and PRDM16 and UCP1 to Dio2.

Linear regression of male LP (left) and NP (right) diets comparing Δ Ct values from

FNDC5 to Δ Ct values from (A) UCP1, (B) PPAR α , and (C) PRDM16 and Δ Ct values

from (D) UCP1 to Δ Ct values from Dio2.

Figure 5. Fetal, Male iBAT UCP1, PPAR α , PRDM16, FNDC5, and PGC-1 α mRNA are not different in the maternal low protein diet in comparison to the normal protein diet. Fetal, male iBAT was isolated at gestation day 18-20, mRNA was collected and thermogenic markers, (A) UCP-1, (B) PPAR α , (C) PRDM16, (D) PGC1 α , and (E) FNDC5 was measured by qPCR and normalized to 18S rRNA. Values are fold change $\pm$ SEM, n=14-15. Significant * = $p \leq 0.05$ between LP and NP.

Figure 6. Placental tissue connected to male fetuses expresses PRDM16, CD137, and FNDC5. Placental tissue connected to male fetuses was isolated at gestation day 18 and RNA was collected to measure thermogenic (PRDM16 and FNDC5) and beige adipocyte (CD137) markers. (A) PRDM16, (B) CD137, (C) FNDC5, and (D) UCP-2 was measured by qPCR and normalized to 18S rRNA. Values are fold change $\pm$ SEM, n=14-15.

Figure 7. Proposed mechanism of maternal low protein diet effect on male offspring. Our data demonstrate that a maternal LP diet increases iBAT thermogenic markers PPAR α , PRDM16, and UCP-1. An increase in FNDC5 was also detected in male, neonatal iBAT and positively correlated with PPAR α , PRDM16 and UCP-1. Additionally, Dio2 was positively correlated with UCP-1 levels in male, neonatal iBAT. Therefore, we propose that male neonates from dams on a LP diet have low birth weight that is due to an increase in thermogenesis by FNDC5 pathways or altered thyroid hormone axis regulation, but not due to a dysregulation in sex hormones or placental thermogenesis.

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Table 1.

Gene	Forward Primer	Reverse Primer	Probe
UCP-1	AGA AGG ATT GCC GAA ACT GTA C	AGA TCT TGC TTC CCA AAG AGG	/56-FAM/AAA GCT GAT /ZEN/ TTG CCT CTG GAT GCC /3IABkFQ/
UCP-2	ATG TGG TAA AGG TCC GCT TC	CAT TTC GGG CAA CAT TGG G	/56-FAM/ATG GTC TTG /ZEN/ TAG GCT TCG ACA GTG C /3IABkFQ/
UCP-3	TGG TGA AGG TCC GAT TTC AAG	CGT TTC TTG TGA TGT TGG GC	/56-FAM/AGG CGA GAG /ZEN/ GAA ATA CAG AGG GAC T /3IABkFQ/
PRDM16	CAG AAA GGG CAA GGA GAG ATA C	GTT GGA GGA GAT GCT GAA TGA	/56-FAM/TAC TTG CAC /ZEN/CTG TAT GGC TGT TCC C/3IABkFQ/
FNDC5	AGG ACA ACG AGC CCA ATA AC	CAT ATC TTG CTT CGG AGG AGA C	/56-FAM/AAG AGT GCA /ZEN/TCA GAG ACC AGC ACC /3IABkFQ/
PPAR α	AGT GTG ATC GAA GCT GCA AG	GCC TTC AGT TTT GCT TTC TCA G	/56-FAM/CCG TTT CCA /ZEN/CAA GTG CCT GTC C/3IABkFQ/
Dio2	CTC CTA GAC GCC TAC AAA CAG	TGC TTC AGG ATT GGA CAC G	/56-FAM/TGA AGA TGC /ZEN/TCC CAA TTC CAG TGT CG/3IABkFQ/
PGC-1 α	ACC AAA CCC ACA GAG AAC AG	GGG TCA GAG GAA GAG ATA AAG TTG	/56-FAM/AAG TCC CAT /ZEN/ACA CAA CCG CAG TCG /3IABkFQ/
CD137 (Tnfrsf9)	TTC TTA CCT TGT TCC TGG CG	CCT CTT GAG CAG TTC TAA CCG	/56-FAM/CCA TTT GGG /ZEN/CAC AGA GAA CCA GAG A/3IABkFQ/

Figure 1.

Figure 2.

Figure 3.

Figure 4.

(A)

(B)

(C)

(D)

Figure 5.

(A)

(B)

(C)

(D)

(E)

Figure 6.

(A)

(B)

(C)

(D)

Figure 7.