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ORIGINAL ARTICLE

Altered adipocyte structure and function in nutritionally programmed microswine offspring

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Adipose tissue (AT) dysfunction links obesity of any cause with cardiometabolic disease, but whether early-life nutritional deficiency can program adipocyte dysfunction independently of obesity is untested. In 3–5-month-old juvenile microswine offspring exposed to isocaloric perinatal maternal protein restriction (MPR) and exhibiting accelerated prepubertal fat accrual without obesity, we assessed markers of acquired obesity: adiponectin and tumor necrosis factor (TNF)-α messenger ribonucleic acid (mRNA) levels and adipocyte size in intra-abdominal (ABD-AT) and subcutaneous (SC-AT) adipose tissues. Plasma cortisol, leptin and insulin levels were measured in fetal, neonatal and juvenile offspring. In juvenile low-protein offspring (LPO), adipocyte size in ABD-AT was reduced 22% ($P = 0.011$ *v.* controls), whereas adipocyte size in SC-AT was increased in female LPO ($P = 0.05$) and normal in male LPO; yet, adiponectin mRNA in LPO was low in both sexes and in both depots ($P < 0.001$). Plasma leptin ($P = 0.004$) and cortisol ($P < 0.05$) were reduced only in neonatal LPO during MPR. In juveniles, correlations between % body fat and adiponectin mRNA, TNF-α mRNA or plasma leptin were significant in normal-protein offspring (NPO) but absent in LPO. Plasma glucose in juvenile LPO was increased in males but decreased in females (interaction, $P = 0.023$); plasma insulin levels and insulin sensitivity were unaffected. Findings support nutritional programming of adipocyte size and gene expression and subtly altered glucose homeostasis. Reduced adiponectin mRNA and adipokine dysregulation in juvenile LPO following accelerated growth occurred independently of obesity, adipocyte hypertrophy or inflammatory markers; thus, perinatal MPR and/or growth acceleration can alter adipocyte structure and disturb adipokine homeostasis in metabolically adverse patterns predictive of enhanced disease risk.

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Introduction

Poor intrauterine growth is associated with increased risk of metabolic and cardiovascular disease later in life;[1–4] accelerated growth during childhood amplifies these risks.[5,6] One potentially powerful amplifier is excess adiposity, which often accompanies the accelerated growth following early growth restriction and which mediates disease via multiple pathways.[7] Obesity reduces protein and transcript levels of adiponectin,[8,9] a powerful adipokine known to enhance insulin sensitivity and fat oxidation as well as to counteract inflammatory and oxidative stressors. Leptin dysregulation in obese states includes resistance to leptin's central appetite suppressing actions while mediating obesity-related central sympathetic activation with attendant adverse cardiovascular impacts.[10] Increased macrophage infiltration into adipose tissue (AT), with systemic release of proinflammatory adipokines [e.g., tumor necrosis factor (TNF) α, IL[6]], promote generalized tissue inflammation and cardiovascular injury.[11] Lastly, the obese state is characterized by increased lipolysis in intra-abdominal adipose tissue (ABD-AT), setting off a cascade of elevated circulating free fatty acids, multi-organ lipid overload and secondary metabolic impairments that include hepatic and skeletal muscle insulin resistance.[12]

A second proposed pathway for developmentally linked increase in cardiometabolic risk in later life is programmed dysregulation of glucocorticoid homeostasis, which may also reduce insulin sensitivity and impair cardiovascular function through the adverse actions of cortisol (see reviews: Clark[13] and Reynolds[14]).

In a recent paper in this journal, we describe growth patterns and body composition of microswine offspring exposed to perinatal maternal protein restriction (MPR).[15] Pregnant sows were randomized to either 14% protein (control normal protein) or 1% protein (low protein) diets

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during the last quarter of gestation and first 2 weeks of lactation. The postnatal diet extension of MPR encompasses the major period of adipogenesis in swine, which occurs later in pigs than in more precocial species. Born with only ~1–2% body fat, piglets require 4 weeks to accrue the 12–15% fat present in human newborns.[16–18] Piglets were weaned at 4 weeks of age to normal growth chow, and followed serially to 3–5 months of age. We found[15] that low-protein offspring (LPO) have reduced fetal and early postnatal growth rates, followed by accelerated prepubertal body growth: accelerated in absolute terms for length (cm/week) but only in relative terms for weight (g/kg body weight/week). By dual-energy X-ray absorptiometry (DEXA) scan at 11 weeks of age (late in the accelerated growth phase), LPO had low-normal % body fat and frankly low body fat:length ratio, while lean tissue was more effectively preserved. Overall, LPO at 11 weeks remained thin (low weight:length) as a result of the accelerated linear growth together with normal absolute gain of both fat and lean tissue mass during prepubertal development. Despite absence of obesity in LPO by every measure, the seven-fold increase in fat mass from 6 to 11 weeks of age in LPO significantly exceeded the five-fold increase in controls. We hypothesized that, even in the absence of overt obesity, the relatively rapid accrual of AT during prepubertal growth could have long-lasting metabolic effects mimicking obesity.

In the present study, we thus asked whether the *rapid accrual* of fat tissue during prepubertal development might mimic the known effects of diet-induced or genetic forms of obesity on adipocyte structure and function, despite the absence of AT excess. These effects include adipocyte hypertrophy, hyperleptinemia, hypoadiponectinemia with reduced adiponectin gene expression and elevated markers of inflammation (see reviews: de Ferranti and Mozaffarin[7] and Engeli *et al.*[9]). Therefore, we tested adipocyte size, AT gene expression (adiponectin and TNF-α), circulating leptin levels and glucose homeostasis in the same offspring described in the recent study. Additionally, we tested whether alterations in the glucocorticoid system were present. Our results demonstrate sex-specific alterations of adipocyte structure, function and adipokine regulation in these non-obese juvenile microswine offspring exposed to perinatal MPR-induced asymmetric growth restriction followed by accelerated prepubertal growth.

Methods

Ethical approval

Experiments were approved by the OHSU Institutional Animal Care and Use Committee under protocol no. A439 (June 2005). Requirements to justify utilization of animal models were fully met and approved; efforts to reduce animal numbers and to refine procedures to minimize pain and distress were also described in detail and approved.

Animal care and experimental design

The data described in this study were derived from the same animals described in a recent paper in this journal,[15] as well as in several other studies.[19–21] Brief general methods and those specific to this study are reported here; for details, see the recent paper.

Time-bred Yucatan microswine sows were maintained from gestation day (GD) 80 out of 113 in the OHSU Animal Care Facility under standardized humidity and temperature, and a 12:12 h light:dark cycle. Pregnant sows were randomized to either a control diet (14% protein/75% carbohydrate) or an isocaloric low-protein diet (1% protein/88% carbohydrate) applied during the last fourth of gestation plus 2 weeks postnatally. At 2 weeks, all sows were returned to a normal diet. Offspring to be studied as juveniles were weaned at 4 weeks to a standard, commercially available juvenile piglet 'grower' diet (Purina Mills Lab Porcine Diet Grower; 20% w/w protein, 4% w/w fat, the latter representing 8.6% of calories). Separate litters were generated in the same manner with offspring sacrificed at near-term fetal (GD 113 out of 115) or neonatal (2 weeks postnatal) stages for study of selected outcomes, including plasma cortisol, leptin, insulin and glucose levels. All viable, non-runted piglets were used in this study; there were no differences in number of offspring per litter or sex ratios of offspring based on maternal diet (for details, see DuPriest *et al.*[15]). N varies by assay due to technical reasons; these are listed in tables and figure legends as appropriate.

Tissue, plasma and urine collection

Juvenile piglets [$n = 16$ normal-protein offspring (NPO; 9 males, 7 females), $n = 17$ LPO (10 males, 7 females); 4 NPO litters, 5 LPO litters] were placed in metabolic cages for 48 h at age 11–12 weeks. Urine collected from 24 to 48 h was centrifuged to clear debris and the cleared urine stored at −20°C until use. Over 14–22 weeks of age, sets of four juveniles each underwent a 3-week study protocol including placement of venous catheters under isoflurane anesthesia (2.0–2.5% isoflurane, occasionally up to 3.0% during the first minutes of induction, with an oxygen flow rate of 2.0 l/min) and collection of venous blood in ethylenediaminetetraacetic acid (EDTA) or sodium heparin from conscious pigs; plasma was stored at −80°C until analysis. After 1–2 weeks recovery, organs, plasma and tissues were harvested under isoflurane anesthesia. Subcutaneous adipose tissue (SC-AT) from the ventral abdomen and ABD-AT from the lateral interior abdominal wall were quickly harvested, snap-frozen in liquid nitrogen, and stored at −80°C until use. Separate aliquots of ATs were fixed in formalin, then paraffin embedded. Neonatal offspring studied at 2–3 weeks of age underwent a similar procedure for blood collection; since pigs develop AT primarily postnatally, AT was too sparse to collect in NPO and LPO neonates.[16,17]

To obtain samples from near-term fetal animals, pregnant sows at 113 days of gestation were anesthetized by inhaled

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isoflurane (dose as described above). Piglets were delivered by Cesarean section one at a time and quickly sacrificed by exsanguination via cardiac puncture. Blood was collected into EDTA- or heparin-containing tubes, or without anticoagulant as described above. As with neonates, AT was insufficient for sampling in fetal piglets.

Adipocyte size measurements

Two randomly selected formalin-fixed paraffin-embedded ABD-AT blocks and two SC-AT blocks from each animal were cut in 5-μm sections and two non-adjacent slides per block were then stained with hematoxylin. Digital images were captured from a random field in each of four quadrants per section using a Leica microscope with 20× objective and charge coupled device camera. Images were analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) software. Cell areas (μm^2) were measured and averaged for each section; section averages were then averaged within blocks, then across blocks to obtain a single average for each fat depot per animal. All analysis procedures were performed by an individual blinded to the maternal diet and sex of the animal.

Plasma analysis

The Coat-A-Count Cortisol RIA kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) was used to assay plasma (sodium heparin) and urine cortisol in juvenile offspring. Cortisol was extracted from urine samples with dichloromethane prior to assay according to kit instructions. For urine samples, concentrations were multiplied by 24-h volume to yield excretion rate in nmol per day. Urine excretion rates were normalized to lean body mass at 11 weeks. Within-assay coefficient of variability (CV) was 2.45% and between-assay CV was 4.24%. The assay is highly specific for cortisol, with only 0.98% cross-reactivity with cortisone according to kit manual (the assay does cross-react with prednisolone, but this is relevant only in patients given this therapy); the assay range is 27.6–1380 nmol/l, and linearity in the assay range exceeds 92% in all measurements provided by the manufacturer.

Plasma samples collected in EDTA and obtained under anesthesia were analyzed for leptin concentration using the Multi-Species Leptin Radioimmunoassay Kit (Millipore, Inc., Billerica, MA, USA; formerly Linco Research, St Charles, MO, USA) according to the kit instructions; values are reported in human equivalents (HE). Intra-assay CV was 6.5%; interassay CV was 5.8%. The specificity of the assay for porcine leptin is 67% (compared with 100% for human leptin with the same kit) according to manufacturer; the assay range is 1.0–50 ng/ml HE, and linearity in the assay range exceeds 94% in all measurements provided by the manufacturer.

Heparinized plasma samples were analyzed for insulin concentration using the Porcine Insulin RIA kit (Millipore, Inc; formerly Linco Research; used for juvenile offspring) or Human Insulin Specific RIA kit (Millipore, Inc., used for near-term fetal and 2-week neonatal offspring) according to kit instructions. Intra-assay CV for Porcine Insulin RIA was 9.7%; all measurements were performed in a single assay. Intra-assay CV for Human Insulin RIA was 2.4%; all measurements were performed in a single assay. The specificity of both assays was 100% according to the manufacturer; assay ranges were from 2 to 200 μU/ml; linearity in the assay range exceeded 86%. Insulin values were Ln transformed prior to analysis. Quantitative insulin sensitivity check index (QUICKI) values were calculated as 1/[(log fasting plasma insulin) + (log fasting plasma glucose)].[22] Plasma glucose samples were measured by the OHSU Clinical Lab.

Real-time polymerase chain reaction (PCR)

Snap-frozen tissue samples were homogenized in TRIzol (Invitrogen Corporation, Carlsbad, CA, USA), and total ribonucleic acid (RNA) was extracted according to manufacturer instructions. RNA was treated with DNase I (Invitrogen), then reversed transcribed using random hexamer primers (Operon Biotechnologies, Huntsville, AL, USA) and SuperScript II reverse transcriptase (Invitrogen). Resulting cDNA was analyzed in triplicate using the SYBR Green method. Gene-specific primers to swine adiponectin, TNF-α and 18s ribosomal ribonucleic acid (rRNA) were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Primers for glucocorticoid receptor (GR)-α and 11β hydroxysteroid dehydrogenase (HSD1) were adapted for swine, based on those used in human studies.[23] All primers were synthesized by Operon Biotechnologies; see Table 1 for sequences. All amplicon identities were confirmed by visualizing appropriately sized single bands on agarose gels and also by cloning into pGEM-T-Easy vector via cloning system I Kit (Promega) and sequencing using SP6 primer. Amplification reactions were run on an ABI Prism 7500 (Applied Biosystems) with the following protocol: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 90°C for 15 s and 60°C for 60 s. Relative gene expression was calculated by the standard curve method using serial dilutions of a pool of a large subset of samples, and normalized to endogenous controls (18s rRNA). C_t values of 18s rRNA per microgram of RNA were not affected by maternal diet in juvenile offspring (3–5 months).

Statistical methods

For each parameter in juveniles, covariates were identified by stepwise regression analysis (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL, USA) using body weight at harvest, age at harvest, and, where relevant, % body fat at 11 weeks (by DEXA[15]). Adipocyte size (area in μm^2) was analyzed by pooling average cell areas across each slide, then averaging across slides to obtain one mean value for each fat depot per animal. For ABD-AT, using % body fat at 11 weeks and body weight at harvest[15] as covariates in a General Linear Model, values were analyzed using maternal diet and sex as factors. For real-time PCR assays,

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Table 1. *Real-time PCR primer sequences*

Transcript/accession no.	Forward (5′–3′)	Reverse (5′–3′)	Amplicon length
18s rRNA AF179868	CAGCAGCCGCGGTAATTC	ACGAGCTTTTTAACTGCAGCAA	64
GR-α[a] NM001008481	CATTGTCAAGAGGGAAGGAAACTC	GATTTTCAACCACATCATGCATGGA	95
11βHSD1[a] NM214248	GAATATTCAGTGACCAAGGTCAA	TAATTTCCAGGGCGCATTCTT	139
Adiponectin AY672882	GGCCGTGATGGCAGAGAT	CCCTTAGGACCAGTAAGACCTGTATC	81
TNF-α X54859	CTCTGGCCCAAGGACTCAGA	GTGGGCGACGGGCTTAT	60

PCR, polymerase chain reaction; rRNA, ribosomal ribonucleic acid; GR, glucocorticoid receptor; HSD, hydroxysteroid dehydrogenase; TNF, tumor necrosis factor.

[a] Primer sequences for GR-α and 11βHSD1 were adapted for the pig from previously described human primers.[23]

results are shown as a ratio of gene-of-interest to endogenous control (18s rRNA). These ratios were Ln transformed, then analyzed by analysis of variance (ANOVA) using maternal diet, sex and AT depot (ABD-AT *v.* SC-AT) as factors. Ln-transformed plasma leptin and insulin values, as well as raw plasma glucose, plasma cortisol and QUICKI values were analyzed within each age group using maternal diet and sex as factors. Data are presented as means ± S.E.M. For all statistical tests, a P-value < 0.05 was considered significant.

Results

Adipocyte size and gene expression in juvenile microswine

After relatively accelerated fat mass accrual over 6–11 weeks (as separately reported[15]), LPO at 11 weeks nonetheless remained thin, showing persistently lower body weight, low-normal % body fat by DEXA scanning and significantly reduced fat mass:body length ratio. We therefore addressed whether this rapid fat accumulation affected adipocyte size and gene expression despite absence of obesity.

Physiologic determinants of adipocyte size in juveniles: % body fat and body weight relationships

In all juveniles ($n = 6$ male NPO, 9 female NPO, 8–9 male LPO, 7 female LPO), ABD-AT adipocyte size was directly related to body weight at harvest ($r^2 = 0.56$, $P < 0.0001$; Fig. 1a), and also to % body fat at 11 weeks ($r^2 = 0.33$, $P = 0.002$; data not shown). Following adjustment for body weight at harvest, ABD-AT adipocyte size retained a significant relationship with % body fat ($r^2 = 0.25$, $P < 0.01$; Fig. 1b), indicating an independent effect that was confirmed by stepwise regression analysis using both terms (full corrected model $r^2 = 0.67$, $P < 0.0001$). In juvenile SC-AT, adipocyte size was directly related only to % body fat at 11 weeks ($r^2 = 0.40$, $P < 0.001$; Fig. 1c). When examined separately, NPO and LPO exhibited similar relationships. To control for these confounding effects, body weight and/or % body fat were included as covariates in the analysis of MPR and sex effects on adipocyte size. Results are presented as the covariate-adjusted values generated within the ANOVA unless otherwise stated. For adipocyte size and gene expression, 'adjusted' ABD-AT values are adjusted for % body fat and body weight at harvest; 'adjusted' SC-AT values are adjusted only for % body fat.

Maternal diet effects on adipocyte size

In ABD-AT, adjusted adipocyte size was reduced ~22% in LPO compared with NPO, with no sex differences observed (adjusted values: 3227 ± 242 μm^2 in LPO *v.* 4159 ± 209 μm^2 in NPO; $P = 0.011$; Fig. 1d). In SC-AT, adjusted adipocyte size was increased in female LPO ($P = 0.05$), but normal in male LPO (Fig. 1e), each as compared with sex-matched NPO controls.

Maternal diet effects on the relationships of AT gene expression with % body fat and body weight

Ln-transformed adiponectin mRNA levels in ABD-AT were directly related to % body fat at 11 weeks in NPO ($r^2 = 0.35$, $P = 0.03$; Fig. 2a); however, there was no such relationship in LPO (Fig. 2b). In SC-AT, Ln-transformed adiponectin mRNA levels were weakly related to % body fat when NPO and LPO data were combined ($r^2 = 0.22$, $P = 0.01$; data not shown); however, no relationship was observed in either group alone. Ln-transformed adiponectin mRNA levels were not related to body weight at harvest in either AT depot.

Unexpectedly, Ln-transformed TNF-α mRNA levels in SC-AT were inversely related to % body fat at 11 weeks in NPO ($r^2 = 0.50$, $P = 0.005$; Fig. 2c); LPO did not exhibit any correlation (Fig. 2d). TNF-α mRNA levels in SC-AT were unrelated to body weight at harvest, and TNF-α mRNA levels in ABD-AT were unrelated to either body weight at harvest or % body fat at 11 weeks.

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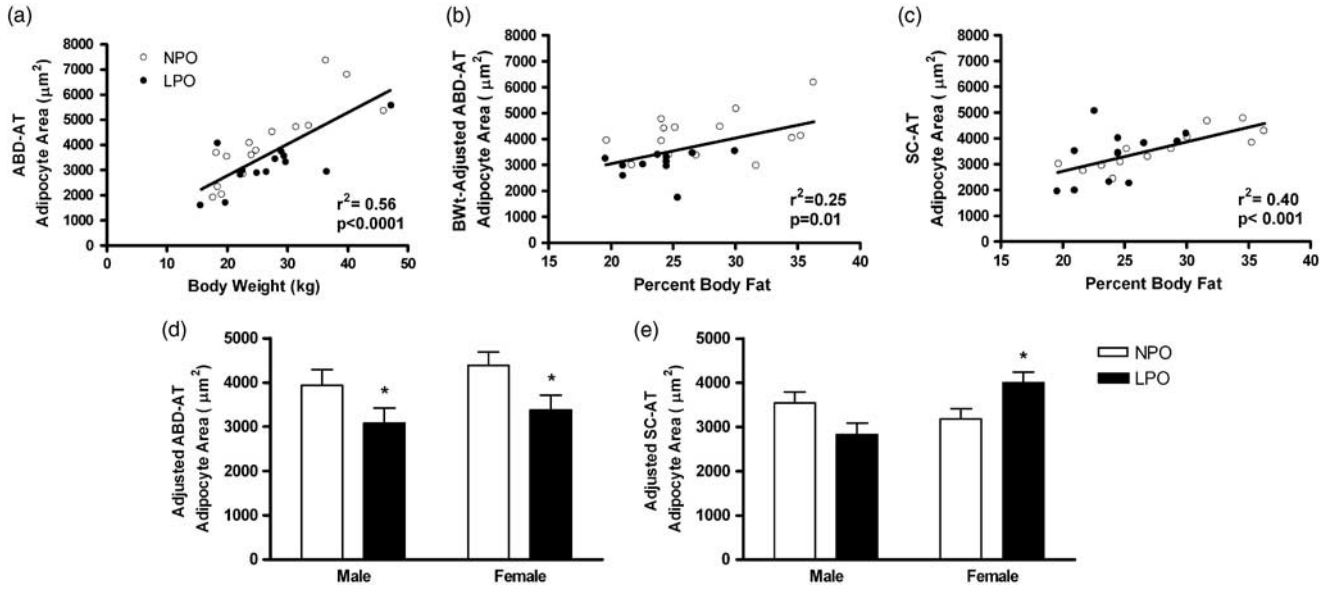


Fig. 1. Adipocyte size in intra-abdominal adipose tissue (ABD-AT) and subcutaneous adipose tissue (SC-AT). Physiological determinants of adipocyte size are shown in panels *a*, *b* and *c*; panels *d* and *e* show effects of maternal diet on adipocyte size after adjusting for relationships shown in *a*, *b* and *c*. (*a*) Relationship between body weight at harvest and ABD-AT adipocyte size (i.e. mean cross-sectional area, in μm^2). (*b*) Relationship between body weight-adjusted ABD-AT adipocyte size and % body fat at 11 weeks. (*c*) Relationship between % body fat at 11 weeks and SC-AT adipocyte size. (*d*) ABD-AT adipocyte size, adjusted for body weight at harvest and % body fat adjusted. (*e*) SC-AT adipocyte size, adjusted for % body fat. Data in panels *d* and *e* are shown as means ± S.E.M. White bars, normal-protein offspring (NPO; $n = 14$; 5 males, 9 females); black bars, low-protein offspring (LPO; $n = 12$; 6 males, 6 females). Maternal diet, $^*P < 0.02$.

Maternal diet effects on AT gene expression

Adiponectin mRNA in juvenile LPO was lower in both fat depots and in both sexes *v.* NPO ($P < 0.001$ in sex-combined analysis; Fig. 2e and 2f). However, the reduction in LPO females (69 ± 5%) was significantly greater than the reduction observed in males (36 ± 10%; $P = 0.02$ for sex by diet interaction). TNF-α mRNA was significantly lower in female LPO ($P = 0.03$), but statistically unchanged in male LPO compared with sex-matched NPO in both fat depots (Fig. 2g and 2h). Both adiponectin and TNF-α mRNA levels in ABD-AT were higher than in SC-AT ($P < 0.001$ for each; Fig. 2e–2h). Neither adjusted ABD-AT adipocyte size nor adjusted SC-AT adipocyte size was correlated with mRNA levels of adiponectin or TNF-α in either AT depot.

Metabolic indices in near-term fetal, neonatal and juvenile microswine

Circulating markers of metabolic function were assessed in NPO and LPO at three stages of development: near-term fetal (GD 113 out of 115), neonatal (2 weeks postnatal) and juvenile (3–5 months postnatal); see Table 2 for N and sex distributions for each age group.

Plasma leptin

In near-term fetal offspring, no differences in plasma leptin levels were observed in LPO *v.* NPO. At 2 weeks of age, still during MPR, plasma leptin levels were reduced in LPO compared with NPO ($P = 0.004$; Fig. 3a). In juveniles (NPO and LPO combined), plasma leptin levels were directly related to % body fat at 11 weeks ($r^2 = 0.239$, $P = 0.007$). However, when NPO and LPO were analyzed separately, only NPO exhibited a significant relationship between plasma leptin and % body fat ($r^2 = 0.640$, $P = 0.0003$; Fig. 3c); LPO did not show any relationship (Fig. 3d). With or without adjustment for % body fat, plasma leptin levels in juvenile LPO showed no difference compared with NPO. Overall, juvenile females had higher leptin levels than juvenile males ($P = 0.04$; Fig. 3b). After pooling NPO and LPO, plasma leptin levels were positively correlated with unadjusted adipocyte size in ABD-AT ($r^2 = 0.16$, $P = 0.034$) and in SC-AT ($r^2 = 0.26$, $P = 0.008$). However, leptin was not related to adjusted adipocyte sizes in either ABD-AT or SC-AT.

Glucose homeostasis

In juveniles, fasting plasma glucose levels were higher in male LPO, but lower in female LPO, compared with sex-matched controls, though within-sex comparisons were not significant (maternal diet by sex interaction, $P = 0.03$; Fig. 4). Juvenile LPO of both sexes had unchanged fasting plasma insulin levels (Table 2). Similarly, QUICKI values, an index of insulin sensitivity, were unchanged in juvenile male and female LPO (Table 2). Glucose levels in near-term fetal and neonatal offspring, as well as plasma insulin levels and QUICKI values in neonatal offspring, were not affected by

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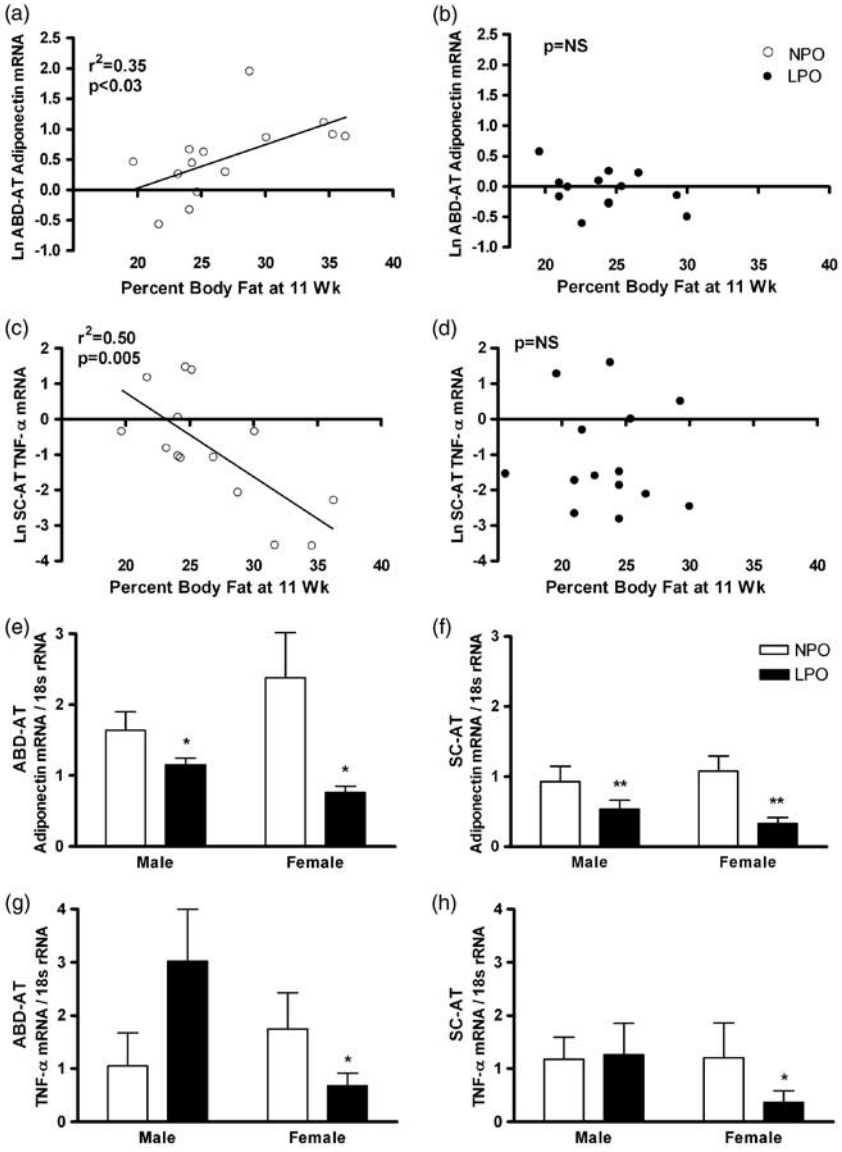


Fig. 2. Adipose tissue adiponectin and tumor necrosis factor (TNF)-α messenger ribonucleic acid (mRNA) expression in intra-abdominal adipose tissue (ABD-AT) and subcutaneous adipose tissue (SC-AT) in juvenile offspring. Relationship between % body fat at 11 weeks and ABD-AT adiponectin or TNF-α mRNA levels is shown for normal-protein offspring (NPO) (*a*, open circles) or low-protein offspring (LPO) (*b*, black circles). Relationship between % body fat at 11 weeks and SC-AT TNF-α mRNA levels is shown for NPO (*c*, open circles) or LPO (*d*, black circles). Adiponectin mRNA levels in ABD-AT (*e*) and SC-AT (*f*); and TNF-α mRNA levels in ABD-AT (*g*) and SC-AT (*h*) are normalized to 18s ribosomal ribonucleic acid (rRNA) and shown as means ± S.E.M. White bars, NPO ($n = 15$; 6 males, 9 females); black bars, LPO ($n = 16$; 9 males, 7 females). Maternal diet, $^{*}P = 0.03$; maternal diet, $^{**}P < 0.002$.

either maternal diet or sex (Table 2; insulin levels were not determined in near-term fetal offspring.)

Indices of glucocorticoid system activity in fetal, neonatal and juvenile microswine

Plasma and tissue parameters related to glucocorticoid function were studied in subsets of offspring. (See Table 2 for *n* and sex distribution for each plasma or urine analysis.)

Plasma cortisol and urinary cortisol excretion

In 2-week-old neonatal LPO during MPR, plasma cortisol was lower than in NPO with no significant sex differences observed (Fig. 5b and Table 2). There was a general trend toward decreased plasma cortisol levels in LPO offspring of all ages, but this decrease was significant only in the 2-week neonates during MPR (Fig. 5a and Table 2).

In juvenile offspring, 24-h urinary cortisol excretion, both absolute (nmol/day) and normalized to lean mass (nmol/day/kg),

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Table 2. *Plasma and urine metabolic indices*[a]

	Fetal		Neonatal		Juvenile	
	NPO	LPO	NPO	LPO	NPO	LPO
Plasma insulin (ng/ml)	–	–	7.98 ± 1.27 (5, 4)	6.16 ± 0.70 (5, 3)	4.15 ± 0.76 (7, 9)	4.92 ± 1.07 (10, 7)
QUICKI value[b]	–	–	0.33 ± 0.006 (5, 4)	0.34 ± 0.006 (5, 3)	0.39 ± 0.009 (7, 9)	0.39 ± 0.011 (10, 7)
Plasma cortisol (nM)	382 ± 38 (11, 6)	303 ± 38 (7, 12)	605 ± 1.9 (5, 4)	502 ± 2.7[c] (5, 3)	450 ± 88 (6, 6)	400 ± 44 (9, 8)
Urinary cortisol excretion						
Absolute (nmol/day)	–	–	–	–	65.5 ± 7.4 (5, 4)	53.9 ± 6.8 (10, 7)
Normalized (nmol/day/kg)	–	–	–	–	6.32 ± 0.84 (3, 4)	5.57 ± 0.75 (8, 6)

NPO, normal-protein offspring; LPO, low-protein offspring; QUICK, quantitative insulin sensitivity check index.
[a] Values are shown as means ± S.E.M. with *n* shown as (male, female) below.
[b] QUICKI is calculated as 1/[(log fasting plasma insulin) + log (fasting plasma glucose)].
[c] $P < 0.05$ compared with 2-week-old NPO.

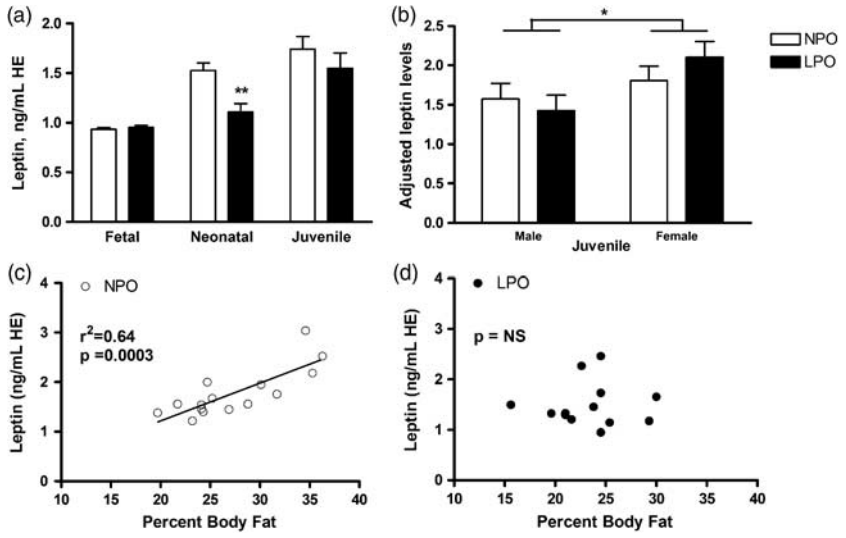


Fig. 3. Fasting plasma leptin concentrations in fetal, neonatal and juvenile offspring. Fasting plasma leptin levels in near-term fetal offspring [gestational day 113 out of 115; $n = 13$ normal-protein offspring (NPO); 14 low-protein offspring (LPO)]; 2-week-old neonatal offspring ($n = 9$ NPO; 8 LPO); and juvenile offspring ($n = 16$ NPO; 17 LPO) are shown as means ± S.E.M. (*a*). Plasma leptin levels (adjusted for % body fat) are shown for juvenile male ($n = 7$ NPO; 10 LPO) and female offspring ($n = 7$ NPO; 7 LPO) as means ± S.E.M. (*b*). White bars, NPO; black bars, LPO. Sex, $^{*}P < 0.05$; maternal diet, $^{**}P < 0.005$. Relationships between % body fat and plasma leptin are shown for NPO (*c*, open circles) or for LPO (*d*, black circles). HE, human equivalents.

was similar between LPO and NPO (Table 2). A weak positive correlation was observed between 24-h cortisol excretion and % body fat at 11 weeks ($r^2 = 0.20$, $P < 0.05$; Fig. 5d), which remained significant after adjusting for lean body mass ($r^2 = 0.21$, $P < 0.04$; Fig. 5e). No correlation was observed between plasma cortisol level and % body fat (Fig. 5f).

AT glucocorticoid system components in juvenile offspring subset

In juvenile ABD-AT and SC-AT, MPR had no effect on mRNA levels of 11βHSD1 or GR-α. Females ($n = 5$ NPO, $n = 7$ LPO) had lower 11βHSD1 mRNA levels compared with males ($n = 5$ NPO, $n = 9$ LPO) in both ABD-AT (Fig. 6a; $P = 0.002$) and SC-AT (Fig. 6b; $P = 0.02$).

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Discussion

Growth during prenatal and postnatal life has been shown to influence risk of cardiovascular and metabolic disease later in life,[1–4] but the mechanisms involved are not yet clear. In the present study, we generated asymmetric growth restriction in microswine offspring using perinatal isocaloric MPR applied from 0.75 gestation through 2 weeks postnatally. The postnatal MPR extension encompasses the major period of adipogenesis in swine. We show that, despite absence of obesity,[15] AT structure and function are nevertheless altered during the late prepubertal period following perinatal MPR and the subsequent accelerated growth. Major findings in LPO include depot- and sex-specific changes in adipocyte size, most prominently reduced adipocyte size in abdominal fat depots; depot-independent reductions in adiponectin mRNA levels (69 ± 5% reduction in LPO females, 36 ± 10% in LPO males) independently of adipocyte size; altered relationships between % body fat and adipokines; and early signs of sex-dependent glucose homeostasis dysregulation.

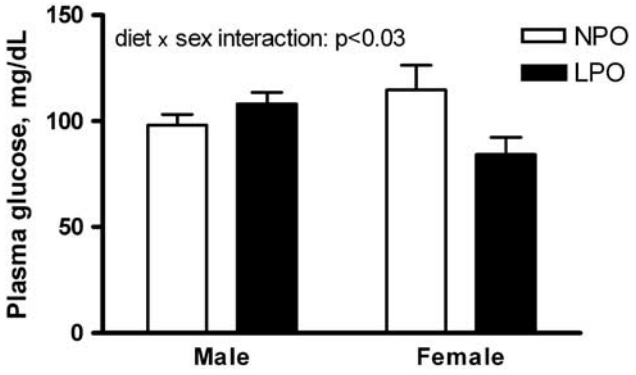


Fig. 4. Plasma glucose levels in juvenile offspring. Fasting plasma glucose levels in juveniles are shown as means ± S.E.M. White bars, normal-protein offspring (NPO; $n = 16$; 7 males, 9 females); black bars, low-protein offspring (LPO; $n = 17$; 10 males, 7 females). Maternal diet by sex interaction, $^*P < 0.03$.

Swine are suited for study of cardiometabolic disease because of their close physiological, anatomical and genomic similarities to humans.[24,25] One important difference is the lack of brown AT in swine,[26] partially attributed to deletion of exons 3–5 in the gene for uncoupling protein (UCP)1 with loss of UCP1 function.[27] Another distinction between swine and humans is the relatively late development of white AT in swine: piglets are born with only ~1–2% body fat,[16,17] requiring 3–4 weeks to acquire the 12–15% body fat typical of the human newborn.[18] Our postnatal extension of MPR was designed to encompass this important phase of adipogenesis.

The juvenile LPO described in this study were previously reported to exhibit rapid accrual of both fat and lean tissues over ages 6–11 weeks, as evidenced by low tissue masses at 6 weeks, and normal tissue masses at 11 weeks.[15] The absence of obesity may in part reflect the relatively low-fat postweaning diet consumed by the offspring, which could mitigate or delay programmed obesity. In a study of human children, obese

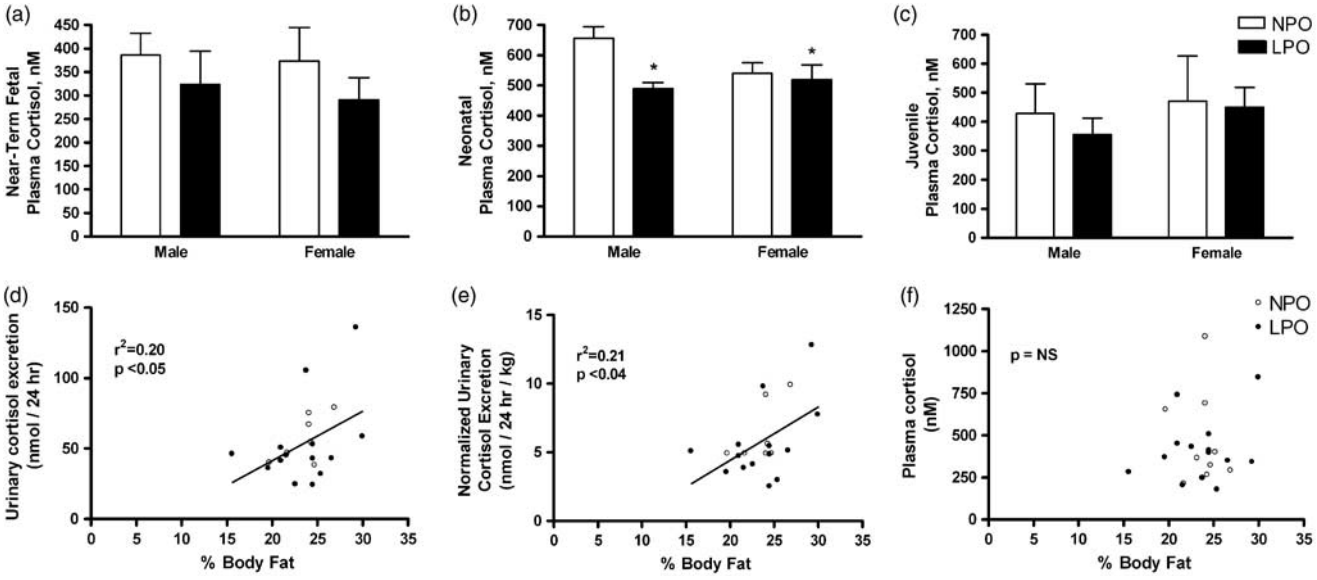


Fig. 5. Plasma cortisol and urinary cortisol excretion in fetal, neonatal and juvenile offspring. Fasting plasma cortisol levels (nM) in near-term fetal [*a*; $n = 11$ normal-protein offspring (NPO) males, 5 NPO females, 7 low-protein offspring (LPO) males, 12 LPO females], 2-week-old neonatal (*b*; $n = 5$ NPO males, 4 NPO females, 4 LPO males and 3 LPO females) and juvenile (*c*; $n = 5$ NPO males, 5 NPO females, 9 LPO males, 9 LPO females) pigs are shown as means ± S.E.M. White bars, NPO; black bars, LPO. Maternal diet, $^*P < 0.05$ with no significant sex by diet interaction. Correlations between 24-h urinary cortisol excretion and % body fat are shown for absolute values (nmol/day, *d*) and for cortisol excretion normalized to lean body mass at 11 weeks (nmol/day/kg, *e*). The scatter plot showing lack of correlation between plasma cortisol level (nM) and % body fat is also shown (*f*).

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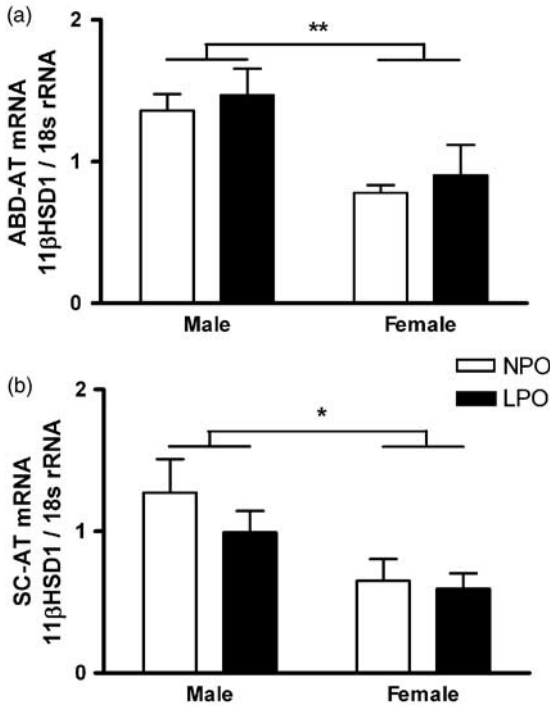


Fig. 6. Adipose tissue 11β hydroxysteroid dehydrogenase (HSD1) messenger ribonucleic acid (mRNA) levels in juvenile offspring. Levels of mRNA for 11βHSD1 are shown for intra-abdominal adipose tissue (ABD-AT) (*a*) and subcutaneous adipose tissue (SC-AT) (*b*) as means ± S.E.M. of values normalized to 18s ribosomal ribonucleic acid (rRNA). White bars, normal-protein offspring (NPO; $n = 10$; 5 males, 5 females); black bars, low-protein offspring (LPO; $n = 16$; 9 males, 7 females). Sex, $^{**}P < 0.002$; sex, $^{*}P < 0.02$.

subjects displayed an earlier and more rapid increase in adipocyte size and adipocyte number.[28] We therefore asked whether, despite absence of excess adiposity, the relatively rapid accrual of AT in LPO (approximately seven-fold increase *v.* five-fold in NPO[15]) would yield adipocyte hypertrophy, as was observed in a study of low-birthweight mice with subsequent catch-up growth,[29] and/or altered adipokine regulation.

We first confirmed in all pigs that both % body fat and body weight were significant physiologic influences modulating ABD-AT adipocyte size, whereas only % body fat influenced SC-AT adipocyte size. This is supported by human data showing that, in adult SC-AT, adipocyte size is correlated with total and % body fat.[30] Unexpectedly, despite adjustment for these physiologic relationships, and despite the greater fold-increase in LPO fat mass,[15] the still lighter, thinner juvenile LPO were found to have *smaller* adipocytes in abdominal AT, rather than the predicted adipocyte hypertrophy. This observation, together with the overall normal % body fat, is compatible with an increase in adipocyte number in LPO ABD-AT. However, because we could not quantify ABD-AT volume independently of total truncal fat by DEXA scanning, we cannot estimate adipocyte number. Nonetheless, adipocyte size was clearly reduced in LPO ABD-AT in both sexes. Moreover, the impact of MPR is depot specific: maternal diet did not affect SC-AT adipocyte size in LPO males, and caused mild SC-AT adipocyte hypertrophy in LPO females. Our ABD-AT data mirror findings in a rat MPR study, which showed decreased adipocyte size in offspring epididymal (visceral) fat pads following maternal low protein diet.[31]

We further investigated whether rapid fat accrual in juvenile LPO induces key metabolic changes that typically accompany fat gain, that is, low adiponectin transcription, increased TNF-α transcription and high plasma leptin levels (see review: de Ferranti and Mozaffarian[7]). In diet-induced and genetic obesity studies, which compare normal *v.* obese states in adults, adiponectin protein and transcript levels[9] decrease with increasing adipocyte size.[32] Based on the reduced ABD-AT adipocyte size observed in LPO, one might expect increased adiponectin transcript levels, specifically in the ABD-AT depot. However, these non-obese juvenile LPO had *reduced* adiponectin mRNA levels in both ABD-AT and SC-AT depots without relationship to adipocyte size and in the absence of inflammatory markers, a pattern distinct from that observed in acquired obesity. This suggests that MPR and/or the consequent accelerated prepubertal growth program persistent changes in some unidentified regulator of adiponectin transcription and/or its mRNA stability. We were unable to measure adiponectin protein levels; however, in both human obesity[33] and rodent obesity models,[9] adiponectin transcript and protein levels are reduced in parallel, suggesting a transcriptional mechanism for hypoadiponectinemia.

Adiponectin transcription can be suppressed by multiple mediators, including insulin,[34] interleukin-6,[35] TNF-α,[36] angiotensin II,[37] sympathetic nervous activity[38] and increased fat mass.[8] In juvenile LPO reported here, we have excluded increased basal insulin level, decreased insulin sensitivity, increased TNF-α and increased fat mass[15] as driving factors for suppressing adiponectin mRNA. Recent feeding studies in mice genetically prone to obesity showed that increased energy intake (calories), but not fat intake or fat mass *per se*, reduced adiponectin gene transcription, possibly via peroxisome proliferator-activated receptor α.[33] In the recent manuscript, we show either low (female) or normal (male) total energy intake in LPO; however, since feed intake per gram of body weight is increased, we cannot exclude a potential role of *relatively* increased calorie intake[15] in suppression of adiponectin mRNA. If this proves relevant, it suggests a novel and potentially powerful mechanism by which accelerated growth enhances cardiovascular/metabolic disease risk following nutritional programming.

Evidence for a role of adiponectin in nutritionally programmed metabolic dysfunction in humans is still controversial. Circulating adiponectin has been shown in studies of children born small for gestational age (SGA) to be either low[39–44] or high,[45,46] although effects may be gender- and/or age specific.[47,48] On the other hand, regardless of starting

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level of adiponectin in SGA children early on, 'catch-up' growth is inversely related to adiponectin levels later in childhood.[43,49–53] The effect of SGA to reduce adiponectin levels persists into adulthood, even without full catch-up growth.[54] This supports the prior suggestion that excess energy intake may suppress adiponectin transcription and potentially explain the propensity of accelerated growth to exacerbate later life disease.

Adipokine relationships with % body fat were disrupted in LPO as compared with controls. Interestingly, in control pigs, the inverse relationship between % body fat and adiponectin levels typically observed in diet-induced or genetic obesity[8] was not present; instead, a positive relationship between these factors was seen in ABD-AT of NPO controls. This may reflect the normal physiologic relationship in the still-developing AT of non-obese juveniles. The inverse relationship previously reported has been largely based on comparing normal and obese subjects who are fully mature, in contrast to the growing pigs studied in this study. In any case, it is clear that LPO lack this normal relationship. Moreover, whereas juvenile NPO did exhibit the expected positive relationships between % body fat and, respectively, plasma leptin levels and SC-AT TNF-α mRNA levels, these relationships were again disrupted in LPO, suggesting a generalized disturbance in the regulation of adipocyte function. In support of the dysregulated adipokine/body fat relationships, microarray of visceral fat of rat offspring exposed to MPR showed *reduced* transcription of 48 inflammation-related genes despite increased visceral adiposity.[55]

AT dysfunction in adults is associated with dysregulated glucose homeostasis and insulin resistance. Our study showed no disruption in insulin production or sensitivity, but plasma glucose levels were altered in a sex-dependent manner. Given that the animals were studied as prepubertal juveniles while on a low-fat diet, it is not surprising that pancreatic function and insulin target tissue sensitivity are still intact. In support of this, a recent study of aged female sheep showed dysregulated glucose homeostasis upon short-term high calorie intake, but not on a normal diet.[56] The altered glucose homeostasis observed in males, together with the reduced adiponectin gene expression, support the potential for development of insulin resistance in adulthood with its attendant risks of hypertension and Type II diabetes mellitus.

Because of the frequently observed disruption in the hypothalamic–pituitary–adrenal (HPA) axis or its peripheral components following maternal protein[57–63] or nutrient restriction,[64–67] we also tested whether late gestation/early lactation MPR affects glucocorticoid homeostasis in the microswine MPR model. Plasma cortisol levels were low at 2 weeks in LPO, but this effect did not persist. No other changes in glucocorticoid production or activity were observed, in contrast to what has been observed in many studies in other species[58,62,68–70] and in spontaneous low-birthweight pigs.[71] We believe this difference is due to the timing of exposure: the critical window of development of the HPA axis may have closed in swine prior to our application of MPR. Alternatively or additionally, swine may respond differently to stress than rodents do, as suggested by Weaver *et al.*[72] in their study on neonatal handling of young boars, and these differences may yield different results depending on which HPA axis measurements were performed.

The data reported here support the concept that perinatal MPR in microswine, with its associated early asymmetric growth restriction and programmed acceleration of prepubertal growth over 6–11 weeks of age, leads to altered adipose structure and gene expression, as well as to functional dysregulation of adipokines independently of the development of obesity or adipocyte hypertrophy. Specifically, MPR and/or accelerated postweaning growth cause(s) reduced ABD-AT adipocyte size, reduced adiponectin mRNA levels and disrupted relationships between % body fat and the adipokines adiponectin, leptin and TNF-α, in each case by mechanisms distinct from those operating in human or rodent forms of obesity. MPR may program the reduction of adiponectin mRNA levels while exerting depot-specific effects on adipocyte size regulation and disconnecting adipocyte leptin and adiponectin from body fat cues. Adiponectin has favorable effects in multiple tissues, that together optimize energy homeostasis, whereas adiponectin deficits contribute to the multiorgan dysfunction associated with metabolic syndrome. Because of these metabolically favorable actions of adiponectin, we propose that a programmed dysregulation of adipocytes, including reduction of adiponectin mRNA, may constitute an important link between early-life nutrient deprivation and later risk of cardiovascular and metabolic disease.

Acknowledgments

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