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Maternal protein restriction during pregnancy and lactation alters central leptin signalling, increases food intake, and decreases bone mass in 1 year old rat offspring

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SUMMARY

The effects of perinatal nutrition on offspring physiology have mostly been examined in young adult animals. Aging constitutes a risk factor for the progressive loss of metabolic flexibility and development of disease. Few studies have examined whether the phenotype programmed by perinatal nutrition persists in aging offspring. Persistence of detrimental phenotypes and their accumulative metabolic effects are important for disease causality. This study determined the effects of maternal protein restriction during pregnancy and lactation on food consumption, central leptin sensitivity, bone health, and susceptibility to high fat diet-induced adiposity in 1-year-old male offspring. Sprague-Dawley rats received either a control or a protein restricted diet throughout pregnancy and lactation and pups were weaned onto laboratory chow. One-year-old low protein (LP) offspring exhibited hyperphagia. The inability of an intraperitoneal (i.p.) leptin injection to reduce food intake indicated that the hyperphagia was mediated by decreased central leptin sensitivity. Hyperphagia was accompanied by lower body weight suggesting increased energy expenditure in LP offspring. Bone density and bone mineral content that are negatively regulated by leptin acting via the sympathetic nervous system (SNS), were decreased in LP offspring. LP offspring did not exhibit increased susceptibility to high fat diet induced metabolic effects or adiposity. The results presented here indicate that the programming effects of perinatal protein restriction are mediated by specific decreases in central leptin signalling to pathways involved in the regulation of food intake along with possible enhancement of different CNS leptin signalling pathways acting via the SNS to regulate bone mass and energy expenditure.

Keywords

areal bone density; central leptin signalling; diet protein restricted; hyperphagia; programming

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INTRODUCTION

The control of body weight is fundamentally dependent on the balance between food intake and energy expenditure. Both these processes are regulated by a diverse set of physiological systems and their exceptionally complicated interactions. Food intake is regulated by central pathways and peripheral hormones. Central regulation of appetite is primarily located in the hypothalamus and is mediated by neuropeptides expressed in the neurons of the arcuate nucleus and their projections to the paraventricular nucleus and the lateral area of the hypothalamus.¹ The primary neuropeptides include the orexigenic neuropeptide Y and agouti related peptide and the anorexigenic peptides, pro-opiomelanocortin and cocaine and amphetamine related transcript. Peripheral regulation of appetite occurs through a variety of hormones that are produced in the fat cells (leptin), the gastrointestinal tract (ghrelin), and the pancreas (insulin) and act through specific receptors located in discrete nuclei of the hypothalamus.² Leptin, the best characterized of these hormones, is a 16 kDa protein secreted by the adipose tissue in direct proportion to fat mass. Leptin is transported across the blood brain barrier and elicits its actions by binding to the long and signalling isoform of the leptin receptor (OB-Rb) that is expressed in discrete regions of the central nervous system (CNS).² Leptin's effects on appetite is completely accounted for by its actions in the CNS where it modulates the balance between orexigenic and anorexigenic neuropeptides.^{1,3}

In rodents, development of the hypothalamic appetite regulatory network begins around day 15 of intrauterine life and is fully mature during neonatal life.^{4,5} Therefore, the integrity of the appetite regulatory network is vulnerable to disruptions during early life. Consistent with this idea, a number of studies have shown that maternal calorie or protein restriction during pregnancy and lactation programs hyperphagia in adult offspring.^{6–10} Additionally, when these offspring are weaned on to a high fat diet they exhibit enhanced susceptibility to adiposity as adults.^{7,11} Most of these studies examining the effects of the perinatal nutrition environment on appetite and body composition are conducted in young adult offspring. Aging constitutes an established risk factor for the progressive loss of metabolic flexibility and the development of adiposity. However, few studies have examined whether the phenotype programmed by perinatal nutrition persists in aging offspring. Persistence of such programmed and detrimental phenotypes and their accumulative metabolic effects is more important for disease causality. Therefore, the primary objective of the current study was to examine the effect of maternal protein restriction during pregnancy and lactation on appetite, metabolic profile, body composition, and susceptibility to high fat diet (HFD)-induced adiposity in middle aged, 1 year old rat offspring. In view of the important role of leptin in regulating appetite and body composition, it was also determined if these long term effects of a perinatal low protein diet were mediated through programmed alterations in central leptin signalling.

In a related development, a growing number of rodent studies have shown that maternal calorie or protein restriction during pregnancy and lactation programs bone health in the offspring.^{12–15} In adults, constant bone mass is a homeostatic function where bone formation is finely balanced by bone resorption.¹⁶ Interestingly, this homeostasis is partially regulated by central leptin signalling.¹⁷ A majority of the studies examining the effect of perinatal protein restriction on bone health have been conducted in young offspring. Aging

is associated with an increased risk of osteoporosis due to deterioration in the homeostasis of bone mass. However, few studies have examined whether the decreased bone mass programmed by perinatal protein restriction persists in aging offspring. Therefore, the second objective of the current study was to determine the effects of maternal protein restriction during pregnancy and lactation on bone health in 1 year old male offspring and to evaluate the role of altered central leptin signalling in mediating these effects. The status of central leptin signalling was assessed by the conduct of a leptin sensitivity test. Appetite was assessed by measuring food intake while body composition and bone health were determined by the measurement of lean and fat mass, bone mineral density, and bone mineral content using dual energy x-ray absorptiometry. Susceptibility of 1 year old low protein offspring to diet-induced adiposity was evaluated by measuring food consumption, body composition and plasma metabolic profile during the metabolic challenge of a high-fat diet.

RESULTS

Phase 1: Characterization of food intake, body composition, bone health, and metabolic profile in 1 year old offspring during laboratory chow feeding

Food intake, body weight and body composition—As shown in Fig. 1a, protein restriction throughout gestation and lactation followed by post-weaning laboratory chow produced hyperphagia as evidenced in higher daily food intake compared to the control offspring. Despite the hyperphagia, one year old low-protein (LP) offspring exhibited lower body weight (Fig. 1b). Dual-energy X-ray absorptiometry (DEXA) scan data showed that LP offspring had lower total body surface area (Table 1). However, body weight normalized lean mass and fat mass were similar between LP and control offspring. LP offspring exhibited lower areal bone mineral density and bone mineral content.

Serum parameters—The serum levels of the anorexigenic hormone leptin were measured to determine its role in mediating the hyperphagia observed in the LP offspring. As shown in Table 2, serum leptin levels in the fed state were similar between control and LP offspring. Fed and fasting glucose and insulin levels were similar between control and LP offspring. Computation of the homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI) indexes showed that insulin sensitivity in 1 year old LP offspring was similar to the controls.

In order to determine the long term effects of perinatal protein restriction on lipid homeostasis, we measured the serum concentrations of various lipids in the fed state when lipid synthesis predominates and in the fasted state when lipid utilization is dominant. Serum levels of triglycerides in the fed state, but not in the fasted state, tended to be lower in the LP offspring. However, there were no differences in fed or fasted levels of cholesterol, free fatty acids, and β -hydroxybutyrate between LP and control offspring. Fasting produced the expected increase in serum free fatty acid and β -hydroxybutyrate levels in both groups.

Phase 2: Comparative leptin sensitivity in control and low protein offspring

Leptin at a dose of 1 mg/kg produced a significant decrease in food consumption in the control male offspring starting at 4 h after the injection and the decrease persisted at all later time points (Fig. 2a). Male LP offspring were leptin insensitive as evidenced by a lack of a reduction in food intake at any time point following the leptin injection (Fig. 2b).

Phase 3: Characterization of food intake, body composition, weight gain, and metabolic profile in 1 year old offspring during high fat diet feeding

Food intake, body weight, and body composition—To determine if the decreased leptin sensitivity in male LP offspring would increase their susceptibility to diet induced obesity, a HFD was administered and daily body weight and food intake measured during a 14-day period. As shown in Table 1, HFD increased total mass in both groups, but LP offspring continued to exhibit lower total body mass and body surface area compared to controls. HFD increased percent fat mass and decreased percent lean mass in both groups but there were no significant differences between control and LP offspring. During HFD feeding, LP offspring continued to exhibit lower areal bone mineral density and bone mineral content compared to control offspring. As shown in Fig. 3, there were no significant differences in HFD mediated weight gain between the control and LP offspring suggesting that LP offspring did not exhibit increased susceptibility to diet-induced obesity. It must be noted that continued administration of the HFD was accompanied by increased variability in percent weight gain. During the HFD feeding period, the daily calorie consumption was similar between the two groups of offspring (Fig. 4).

Serum parameters—As shown in Table 2, HFD-mediated increase in adiposity produced an 85–130% increase in fed state leptin levels in control and LP offspring (main effect of HFD, $P=0.0113$). The HFD did not affect glucose levels, but increased fed and fasted insulin levels in control and LP offspring although statistical significance was observed only in fasted insulin levels. The HFD also decreased insulin sensitivity as evidenced by higher HOMA and lower QUICKI indexes. These results indicate that chronic administration of HFD produced the expected deterioration in metabolic profile in both groups of rats. However, there were no differences in HFD-mediated changes in serum leptin and insulin levels or insulin sensitivity indexes between control and LP offspring.

The serum concentrations of various lipid fuels were measured during HFD feeding. The HFD did not affect serum levels of triglycerides or cholesterol in the fed or fasted state. Consistent with the known effects of HFD in increasing fatty acid oxidation, both control and LP offspring exhibited marked increases in fasted, but not fed, β -hydroxybutyrate levels compared to corresponding levels during laboratory chow feeding. In both groups of animals, HFD increased serum levels of free fatty acids in the fed state. However, in both groups, free fatty acid levels in the fasted state during HFD feeding were markedly lower than corresponding fasting levels during laboratory chow feeding. These results indicate that HFD produced the expected alterations in lipid homeostasis. However, there were no significant differences in HFD-mediated changes in serum free fatty acids or β -hydroxybutyrate levels between control and LP offspring.

DISCUSSION

Compared to controls, one year old male rats subject to protein restriction during gestation and lactation are hyperphagic but have lower body weight, and they exhibit lower bone mineral density and bone mineral content. The plasma metabolic profile of 1 year old LP rats is similar to control animals and they do not exhibit accelerated deterioration of metabolic profile or increased susceptibility to obesity during the chronic metabolic stress of HFD feeding.

Protein restriction throughout gestation and lactation produces hyperphagia in young animals.^{8–10} Earlier studies conducted in our laboratory showed that younger, 28–95 days old litter mates of animals used in the current study also exhibited hyperphagia.¹⁸ The present study conducted in 1 year old rats is possibly the first to document the remarkably persistent effects of protein restriction throughout gestation and lactation on appetite. It is well established that appetite is primarily regulated at the level of the hypothalamus. In rodents, hypothalamic neural pathways governing appetite begin developing in the last week of gestation and projections to adjacent appetite controlling hypothalamic nuclei occur post-natally and fully develop at 15–16 days of age.^{4,5} Therefore, developmental plasticity for the programming of appetite is present throughout gestation and lactation though the final phenotype is critically dependent on the timing of the intervention. Calorie or protein restriction selectively during gestation followed by balanced diet during lactation produces hyperphagia in adult offspring.^{7,9,11,19} In an interesting contrast, calorie or protein restriction administered specifically during lactation results either in normophagia or hypophagia in the offspring.^{20–22} Our findings in 1 year old rats confirm results in younger animals and show that hyperphagia programmed by protein restriction during gestation cannot be reversed by continued protein restriction during lactation. Leptin, signalling via OB-Rb in discrete hypothalamic nuclei, is an established inhibitor of food intake.¹ Results of our leptin sensitivity experiments demonstrate that the hyperphagia in the 1 year old LP offspring is mediated by decreased central leptin signalling. Previous studies conducted in much younger animals also show that hyperphagia imprinted by perinatal nutrition is a result of defective central leptin signalling.^{19,23} More detailed mechanistic studies in these animals demonstrate that the increase in food intake is due to increases in the hypothalamic expression of orexigenic peptides and simultaneous decreases in the expression of anorexigenic peptides with consequent enhancement of downstream appetite signalling pathways.^{19,24,25} A similar mechanism is possibly responsible for the hyperphagia observed in the current study.

This study has also shown that protein restriction throughout gestation and lactation decreases areal bone mineral density and bone mineral content. Previous studies conducted in younger animals, including those using 55 day old litter mates of animals used in the present study, have shown that perinatal protein or calorie restriction decreases bone areal mineral density, mineral content, structure, and strength.^{12–15,18} However, deterioration of bone health and osteoporosis are normally associated with advanced age. The present results demonstrate that, in rodents, the imprinting effects of perinatal protein restriction on bone mass are long lasting and persist at least until 1 year of age. Interestingly, clinical studies report an association between low birth weight (likely due to an adverse intrauterine

environment) and increased susceptibility to osteoporosis in middle aged adults.²⁶ These associations have led to the formulation of a hypothesis implicating maternal diet in the developmental origins of osteoporosis.²⁷ Results from our studies in middle aged, one year old rats lend support to this hypothesis.

In addition to the more established mediators of bone mass like sex steroids, vitamin D, and parathyroid hormone, leptin has recently been identified as a regulator of bone health. Leptin acting primarily via the central nervous system increases sympathetic nervous system (SNS) tone to decrease bone mass.^{17,28} Therefore, in the current study, decreased central leptin signalling in the LP offspring would be expected to increase their bone mineral density and bone mineral content. Studies that have mapped leptin sensitive neural pathways, by chemical lesioning of discrete brain nuclei or more recently by using cre-lox technology, have shown that different leptin signalling pathways are involved in the regulation of food intake and bone mass.^{29–32} Leptin regulates food intake by initially binding to the OB-Rb in the arcuate nuclei of the hypothalamus. It then regulates the balance of orexigenic (neuropeptide Y and agouti-related peptide) and anorexigenic (pro-opiomelanocortin and cocaine and amphetamine related transcript) peptides secreted from discrete neurons in the arcuate nucleus which in turn act through melanocortin 4 receptors in the paraventricular nucleus of the hypothalamus to reduce food intake.¹ In contrast, leptin regulates bone mass by binding to OB-Rb in the brainstem and then inhibiting downstream serotonin signalling to the ventromedial nucleus of the hypothalamus. This relieves serotonin induced suppression of SNS activity to the bone, increased stimulation of beta-2 adrenoreceptors on osteoblasts, increased bone resorption, and decreased bone mass.³² Additionally, these two major leptin signalling neural pathways exhibit differential sensitivity to leptin with the threshold of leptin signalling needed to affect bone mass being lower than that needed to affect food intake.³⁰ It is therefore conceivable that in our LP offspring, the pathway regulating food intake that requires a higher level of leptin signalling is compromised and results in hyperphagia. However, in these offspring, the pathway regulating bone mass that requires a lower level of leptin signalling is intact and possibly even enhanced and accounts for the decrease in their bone mass. Such discordant effects of leptin on appetite and bone mass due to site specific differences in CNS leptin signalling have been demonstrated in the literature.^{29,31} Additional studies will be needed to confirm possible site specific differences in our LP offspring.

Perinatal protein restriction did not affect insulin sensitivity in 1 year old offspring as assessed by HOMA and QUICKI indices. A review of the literature reveals inconsistencies in the effects of maternal protein restriction throughout pregnancy and lactation on insulin sensitivity in aged offspring. Some authors have shown that the paradigm produces insulin resistance and impairment of glucose tolerance in 15–21 month old offspring^{33,34} while other authors have measured whole body insulin sensitivity or used the euglycaemic-hyperinsulinaemic clamps and shown no change in insulin sensitivity in 8–17 month old rats that were subject to perinatal calorie or protein restriction.^{35,36} The reasons for these discrepant findings are unclear. Longevity studies show that rats exposed to protein restriction throughout gestation and lactation exhibit a life span similar to control animals, suggesting an absence of serious pathophysiology in these animals.³³ Our findings that 1 year old low protein offspring are just as insulin sensitive as control offspring support results

of these longevity studies. Perinatal protein restriction did not affect plasma lipid levels in 1 year old offspring. Most studies have examined the effect of perinatal low protein diet on lipid levels in young offspring and there is limited data in older offspring. In support of our findings, a couple of studies have shown that protein restriction throughout pregnancy and lactation does not affect fasting plasma triglyceride, cholesterol, and free fatty acid levels in 8–15 month old offspring.^{37,38}

In our final experiments, we exposed 1 year old animals to the stress of a HFD to unmask possible underlying metabolic defects in LP offspring. A high fat challenge produced the expected weight gain, increase in adiposity, and deterioration of insulin sensitivity and metabolic profile. However, and more importantly, LP offspring did not consume increased amounts of the HFD and did not exhibit enhanced susceptibility to the detrimental effects of HFD exposure. In agreement with our findings, studies that have exposed rats to protein or calorie restriction throughout pregnancy and lactation and then exposed the offspring to a HFD did not report increased susceptibility in these animals to metabolic dysfunction, weight gain, or adiposity.^{39,40} In contrast, rats exposed to calorie or protein restriction only during gestation, followed by balanced diet during lactation, exhibited neonatal “catch up” growth and increased susceptibility to HFD induced obesity in adulthood.^{7,40} Interestingly, a balanced diet during gestation followed by protein or calorie restriction selectively during lactation prevents catch up growth and actually reduces susceptibility to obesity and protects animals from the damaging metabolic effects of HFD.^{6,41,42} Collectively, these results illustrate the critical importance of catch up growth in programming increased susceptibility to the harmful effects of HFD. We recently reported growth trajectories of offspring used in the current study and showed that protein restriction during gestation reduced birth weight and continued protein restriction during lactation prevented catch up growth.¹⁸ The absence of catch up growth in LP offspring in the present study possibly accounts for the absence of increased susceptibility to the metabolic effects of high fat feeding.

Despite the persistent hyperphagia, LP offspring had a lower body weight, normal basal metabolic profile, and did not exhibit increased susceptibility to the detrimental effects of HFD. These observations suggests that LP offspring protect themselves from the negative effects of hyperphagia by expending more energy. Leptin is an established positive regulator of energy expenditure and acts by stimulating SNS activity, although the identity of neural pathways mediating this effect is currently under investigation. Several lines of evidence suggest that leptin binds to OB-Rb in the brainstem and in discrete nuclei of the hypothalamus and enhances peripheral sympathetic flow via their connections to the preganglionic sympathetic neurons located in the intermediolateral columns of the spinal cord.^{3,43} Interestingly, and as is evident from the aforementioned discussion, leptin signalling pathways regulating energy expenditure are distinct from those involved in food intake.^{3,44} Therefore, similar to the SNS mediated regulation of bone mass, it is possible that leptin-sensitive neural pathways regulating energy expenditure via peripheral SNS efferents are also intact and possibly enhanced in LP offspring. Such a hypothesized SNS mediated increase in energy expenditure protects LP offspring from the detrimental effects of hyperphagia and HFD. Partial confirmation of an intact SNS tone in LP offspring is evident in the comparative pattern of HFD mediated changes in the serum levels of free fatty acids and β -hydroxybutyrate between control and LP offspring. HFD feeding enhances SNS

activity, which increases fatty acid oxidation and dramatically increases serum levels of β -hydroxybutyrate, the end product of fatty acid oxidation. In control animals, as expected, an intact SNS system allowed for a marked HFD induced increase in fatty acid oxidation that prevented the normal, fasting mediated increase in serum free fatty acid levels. Interestingly, LP offspring were also able to mount a vigorous increase in fatty acid oxidation in response to HFD exposure as reflected in the dramatic increase in their fasted serum β -hydroxybutyrate levels. This indicates that LP offspring possess a completely functional SNS system. The SNS mediated increase in fatty acid oxidation during HFD exposure abolished the normal rise in fasting serum free fatty acid levels of LP offspring. In support of this conclusion, other authors have also shown that adult rat offspring exposed to protein restriction during gestation and lactation have increased SNS tone, as evidenced by increased SNS firing rates and activity, higher plasma noradrenaline levels, and increased expression of beta-adrenoreceptors in a number of SNS innervated peripheral tissues.^{45–47}

In summary, protein restriction throughout gestation and lactation programs remarkably long term hyperphagia mediated by decreased central leptin signalling to pathways regulating the balance between orexigenic and anorexigenic neuropeptides. Additionally, LP offspring exhibit decreases in bone mass and a lower body weight likely due to increases in energy expenditure. These latter effects of perinatal protein restriction are possibly mediated by simultaneous enhancement of different CNS leptin signalling pathways that act via SNS efferents to increase SNS activity in peripheral tissues.

METHODS AND MATERIALS

Animals and experimental design

The study was approved by the Institutional Animal Care and Use Committee of the University of the Sciences in Philadelphia. Pregnant rats were randomly assigned to be fed a modified version of the AIN 76 A purified diet containing 19% protein (Control group) or its corresponding low protein formulation AIN M76 A containing 8% protein (Low protein group). The diets are isoenergetic and their detailed composition has been previously reported.⁴⁸ Pregnant rats (6–9 per group) were individually housed and fed the assigned diet throughout pregnancy and lactation. At birth, pups were weighed, sexed and randomly culled to 12 pups per dam. At 72 h post-birth, litters were randomly culled to 8 pups (4 males and 4 females) to ensure a standard litter size for each dam. On day 28 post-birth, offspring were weaned onto normal laboratory chow (LabDiet, product number 5001) and kept on this diet until they were 1 year old. At 1 year of age, one male offspring from each litter of both groups was evaluated over the course of three sequential experimental phases.

Phase 1: Characterization of food intake, body composition, bone health, and metabolic profile in 1 year old offspring during laboratory chow feeding—

Phase one was conducted over a 28-day period. Initially, blood samples (200 μ L) were obtained from all animals in the fed state and after a 24-h fast in order to assess basal metabolic profile. Next, animals were briefly anesthetized with isoflurane and subjected to a noninvasive dual-energy X-ray absorptiometry (DEXA) body composition analysis as

described later. Finally, during the last 14 days of phase one, daily body weight and food consumption were measured in otherwise undisturbed animals.

Phase 2: Comparative leptin sensitivity in control and low protein offspring—

In phase two, which lasted 10 days, a leptin sensitivity test was conducted as described later.

Phase 3: Characterization of food intake, body composition, weight gain, and metabolic profile in 1 year old offspring during high fat diet feeding—

In phase three, offspring were fed *ad libitum* a HFD diet for 28 days (D12492, Research Diets, New Brunswick, NJ, USA). Consumption of the HFD and weight gain in the offspring were recorded daily for the first 14 days in otherwise undisturbed animals. On day 15, a DEXA scan was conducted in all offspring. On day 21 of the HFD feeding period, a morning blood sample was collected in the fed state. At the end of the HFD feeding period (day 28), a morning blood sample was collected after a 24 h fast.

All blood samples in phases 1 and 3 were collected in cold aprotinin-lined polypropylene tubes, serum harvested and stored at -80°C .

Determination of leptin sensitivity

Leptin sensitivity was determined as described by Coupe *et al.*²³ Briefly, food was removed at 0900 hours and at 1745 hours offspring were injected intraperitoneally either with sterile saline or leptin solution at a dose of 1 mg/kg. Food was returned to all cages at 1800 hours (lights off) and food consumption measured at 2, 4, 6, 12 and 24 h post injection. Animals were allowed a 5-day recovery period following each injection. Leptin solution was freshly prepared by dissolving leptin (PeproTech, Rocky Hill, NJ, USA) in sterile saline to make a 1 mg/mL solution.

Determination of body composition and bone health

Body composition and bone health was determined by noninvasive dual-energy X-ray absorptiometry (DEXA) scanning. Rats were transiently immobilized with isoflurane anaesthesia during the 2–3 min duration of the scan. Scans were analyzed using a software program adapted for small animals (QDR 4500A; Hologic, Bedford, MA, USA). The scans provided *in vivo* estimates of lean mass, fat mass, total mass, percent lean mass, percent body fat, total body surface area, areal bone mineral density (BMD), and bone mineral content (BMC).

Measurement of serum glucose, lipids and hormones

Serum glucose was measured using a kit supplied by Cayman Chemical (Ann Arbor, MI, USA), while serum triglycerides, β -hydroxybutyrate, free fatty acids, and cholesterol were determined using kits purchased from Wako Chemicals (Richmond, VA, USA). Serum insulin and leptin were determined by enzyme-linked immunosorbent assay (ELISA) using kits provided by Alpco (Salem, NH, USA). The inter-day coefficient of variation for all assays was less than 5%.

Fasting levels of serum glucose and insulin were used to compute two measures of insulin sensitivity that are validated in rats.^{49,50} The homeostasis model assessment of insulin resistance (HOMA) was calculated using the formula: $(FPI \times FPG/22.5)$ where FPI is fasting serum insulin expressed in $\mu\text{U/mL}$ and FPG is fasting serum glucose concentration expressed in mmol/L . The quantitative insulin sensitivity check index (QUICKI) was calculated using the formula: $1/[\log(FPI) + \log(FPG)]$ where FPI was expressed in $\mu\text{U/mL}$ and FPG was expressed in mg/dL .

Statistical analyses

All data are expressed as mean $\pm$ standard error of the mean (SEM). In each experiment, we always used one male pup from each litter. Consequently the offspring sample size for each group never exceeded the number of treated dams for that group. Such a conservative experimental design is recommended for experiments in multiparous species since it minimizes the inflation of the α level and the spurious statistical significance that results as a consequence.^{51,52} All serum and DEXA scan parameters were analyzed by two-way split plot ANOVA with maternal diet (control or low protein) and offspring diet (normal laboratory chow or HFD) as the main factors. Weight gain measurements during HFD feeding were conducted repeatedly on the same group of animals and were therefore analyzed using two-way repeated measures ANOVA with maternal diet and time as the main factors. Wherever appropriate, multiple comparisons were conducted using the Bonferroni *post hoc* multiple comparisons test. Unpaired Student's *t*-test was used whenever there was a direct comparison between two different groups of offspring. In the leptin sensitivity test, food intake following the saline and leptin injections in each group of rats at each interval were not normally distributed and were therefore analyzed using the Wilcoxon Signed Rank test. All statistical tests were conducted at a significance level of 0.05 using SAS software version 9.3 (Cary, NC, USA).

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Fig. 1. Daily average food consumption and body weight in 1 year old (□) control and (■) low protein offspring fed normal laboratory chow. (a) Food consumption and (b) body weight. Results are mean $\pm$ standard error of the mean (SEM); $n = 6-9$. * $P < 0.05$ statistically significant relative to controls.

Fig. 2. Leptin sensitivity in the 1-year-old control and low protein offspring. (a) Control: (□) control saline; (■) control leptin. (b) Low protein: (□) low protein saline; (■) low protein leptin. Results are mean $\pm$ standard error of the mean (SEM); $n = 6-9$. * $P < 0.05$ compared to saline treatment.

Fig. 3. Percent weight gain in 1 year old (○) control and (●) low protein offspring during high fat diet feeding. Percent weight gain was computed relative to the body weight at the start of HFD feeding. Results are mean $\pm$ standard error of the mean (SEM); $n = 6-9$.

Fig. 4. Daily average food consumption in 1-year-old (□) control and (■) low protein offspring during high fat diet feeding. Results are mean $\pm$ standard error of the mean (SEM); $n = 6-9$.

Table 1

Body composition analyses in 1-year-old control and low protein male offspring when fed normal laboratory chow and high fat diet.

Parameter (units)	Control offspring		Low protein offspring	
	Normal laboratory chow	High fat diet	Normal laboratory chow	High fat diet
Total mass (g)	531.7 ± 10.8	605.1 ± 21.7 [†]	422.7 ± 11.6 [*]	480.0 ± 19.7 ^{*†}
Area (cm ²)	85.0 ± 1.7	89.4 ± 2.7	73.3 ± 1.4 [*]	75.7 ± 2.3 [*]
Lean mass (g)	428.5 ± 12.4	413.7 ± 11.9	349.0 ± 19.5	336.0 ± 21.2
% Lean	80.6 ± 1.3	68.7 ± 3.0 [†]	82.4 ± 3.1	70.2 ± 3.9 [†]
Fat mass (g)	87.3 ± 7.0	174.0 ± 23.1 [†]	68.8 ± 6.6	130.7 ± 21.6 [†]
% Fat	16.5 ± 1.3	28.5 ± 3.0 [†]	16.3 ± 1.9	27.0 ± 3.9 [†]
BMD (g/cm ²)	0.186 ± 0.004	0.194 ± 0.003	0.169 ± 0.003 [*]	0.175 ± 0.003 [*]
BMC (g)	15.8 ± 0.4	17.3 ± 0.7	12.4 ± 0.4 [*]	13.3 ± 0.5 [*]

Values are mean standard error of the mean (SEM), $n = 6-9$.

BMD, bone mineral density; BMC, bone mineral content.

^{*} Significantly different from control group within the same dietary treatment (laboratory chow or high fat diet), $P < 0.05$.

[†] High fat diet significantly different from normal laboratory chow diet fed within the same group, $P < 0.05$.

Plasma glucose, lipids and hormone levels in the fed and fasted states and insulin sensitivity indexes in 1-year-old control and low protein male offspring when fed normal laboratory chow and high fat diet.

Table 2

Diet	Control offspring				Low protein offspring			
	Normal laboratory chow		High fat diet		Normal laboratory chow		High fat diet	
Parameter (units)	Fed state	Fasted state	Fed state	Fasted state	Fed state	Fasted state	Fed state	Fasted state
Leptin (ng/mL)	1.38 ± 0.20	ND [‡]	2.56 ± 0.71	ND	1.00 ± 0.13	ND	2.33 ± 0.59	ND
Glucose (mg/dL)	104 ± 8	82 ± 5	98 ± 6	82 ± 5	101 ± 3	87 ± 4	112 ± 5	87 ± 3
Insulin (ng/mL)	1.38 ± 0.29	0.28 ± 0.08	2.33 ± 0.80	0.59 ± 0.10 [‡]	0.91 ± 0.15	0.19 ± 0.03	1.86 ± 0.25	0.57 ± 0.10 [‡]
HOMA	ND	2.34 ± 0.68	ND	4.93 ± 0.92 [‡]	ND	1.71 ± 0.24	ND	5.08 ± 0.92 [‡]
QUICKI	ND	0.38 ± 0.02	ND	0.33 ± 0.01 [‡]	ND	0.39 ± 0.01	ND	0.33 ± 0.01 [‡]
Triglycerides (mg/dL)	305 ± 160	139 ± 38	305 ± 133	138 ± 37	196 ± 52	105 ± 25	176 ± 27	90 ± 17
Cholesterol (mg/dL)	170 ± 73	130 ± 32	198 ± 62	134 ± 27	117 ± 42	112 ± 23	156 ± 31	115 ± 21
Free fatty acids (μmol/L)	430 ± 72	966 ± 76	652 ± 60 [‡]	471 ± 40 [‡]	350 ± 35	1062 ± 61	479 ± 29 [‡]	498 ± 22 [‡]
β-Hydroxybutyrate (μmol/L)	56 ± 12	291 ± 38	42 ± 13	854 ± 57 [‡]	69 ± 12	337 ± 50	27 ± 8	1073 ± 59 [‡]

Values are mean ± standard error of the mean (SEM), *n* = 6–9.

HOMA, Homeostasis model assessment; QUICKI, Quantitative insulin sensitivity check index.

[‡] High fat diet significantly different from normal laboratory chow within the same group (control or low protein) and feed status (fed or fasting), *P* < 0.05.

[‡] Not Determined.