Reductions in adipose tissue and skeletal growth in rat adult offspring after prenatal leptin exposure

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Abstract

Leptin is involved in regulating food intake, energy balance and bone formation. Increasing evidence suggests that leptin is also involved in fetal growth and development. The aim of this study was to determine if increased maternal leptin is followed by changes in body composition, skeletal growth or hormonal regulation in the adult rat offspring.

Pregnant rats were given injections of either human recombinant leptin (3.5 mg/kg, i.p.) or vehicle on days 8, 10 and 12 of gestation. Both genders of leptin-exposed offspring showed significantly reduced adipose tissue

weight at adult age. Skeletal growth and cortical bone dimensions were significantly reduced. Circulating testosterone levels were significantly increased in female leptin-exposed offspring, and male leptin-exposed offspring had significant testicular enlargement. No significant effects were seen on circulating leptin levels or hypothalamic protein levels of the leptin receptor.

The results demonstrate that maternally administered leptin is involved in fetal growth and development, leading to lean offspring with reduced skeletal growth.

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Introduction

Leptin, the product of the *ob* gene, is a 16 kDa hormone produced mainly by adipose tissue. Leptin regulates food intake and energy expenditure by signaling targets in the hypothalamus (Zhang *et al.* 1994, Campfield *et al.* 1995). The *ob/ob* mice, which lack the *ob* gene coding for leptin, are not only obese, but also infertile. Both obesity and sterility can be corrected by exogenous administration of leptin, implying that leptin is important for regulating body weight and reproduction (Campfield *et al.* 1995, Chehab *et al.* 1996). The lack of functional leptin in *ob/ob* mice results in a high bone mass phenotype, preceding the onset of obesity. Intracerebroventricular infusion of leptin in *ob/ob* mice leads to a rapid and massive decrease of bone mass, demonstrating that leptin regulates bone remodeling in these animals through a central mechanism (Ducy *et al.* 2000).

Increasing evidence indicates that leptin may play a role in fetal growth and development, in both humans and rodents. Primate and rodent newborns differ in their developmental stage at parturition. A 1-day-old rat corresponds approximately to a human fetus at around 22–24 weeks of gestation, whereas a 7-day-old rat pup is

equivalent to a full-term human infant (Whitelaw & Thoresen 2000). Furthermore, in rats, fat deposition starts postnatally, whereas human fetuses start to deposit fat during the third trimester (Rayner *et al.* 1997). Despite this difference, leptin levels are elevated in both human and animal pregnancies. In humans there is a positive correlation between umbilical cord leptin and newborn body weight and body mass index (Hassink *et al.* 1997, Helland *et al.* 1998, Herrera *et al.* 2000). Furthermore, leptin and its receptor (ObR) have been identified in placenta, cartilage/bone, hair follicles, lung, adipose tissue and the choroid plexus and leptomeninges of the brain in the murine fetus (Hoggard *et al.* 1997, Chen *et al.* 2000). It is suggested that leptin is involved in the regulation of brain development and the maturation and function of neuroendocrine axes (Ahima *et al.* 1998, 1999, Matsuda *et al.* 1999). Taken together, these findings support a functional role of leptin during development; however, controversies still exist regarding the importance of leptin (Hoggard *et al.* 2001).

Leptin correlates with body fat mass in both humans and rats, and dietary fat source has been shown to regulate serum leptin levels in rats (Cha & Jones 1998, Landt *et al.* 1998, Levine *et al.* 1999). The effects of high leptin levels

among pregnant females on early fetal growth and development are important issues that have not been elucidated yet.

There is growing evidence for the importance of the perinatal period in programming predisposition to diseases in adult life (Marmot 1997, Strauss 1997). Reduced fetal growth is statistically associated with hypertension, insulin resistance and dyslipidemia in adulthood (Barker *et al.* 1993, Valdez *et al.* 1994). Permanent programming by hormones *in utero* or during the early postnatal phase has been extensively described. Neonatal testosterone administration to newborn female rats, corresponding to the androgen secretion peak in newborn male pups, resulted in visceral obesity, increased muscle weight and decreased insulin sensitivity in adults (Nilsson *et al.* 1998). Prenatal dexamethasone exposure resulted in offspring with reduced birth weight, decreased central glucocorticoid receptor expression, hyperinsulinemia and increased blood pressure at adult age (Benediktsson *et al.* 1993, Nyirenda *et al.* 1998).

The aim of the present study was to examine the potential effects of increased maternal exposure to leptin on the fetus and the consequences at adult age. Since leptin is known to be involved in the regulation of body weight and food intake (Zhang *et al.* 1994), bone formation (Ducy *et al.* 2000) and hormonal regulation (Heiman *et al.* 1997, Sivitz *et al.* 1997, Carro *et al.* 1999, Caprio *et al.* 2001), these parameters were studied in adult rat offspring exposed to high levels of leptin during early gestation.

Materials and Methods

Animals

Timed-pregnant nulliparous Wistar rats were purchased from B&K Universal (Sollentuna, Sweden) and housed under controlled conditions (temperature 21–22 °C, humidity 55–65%, light on from 0500 to 1900 h) with one animal in each cage until parturition. Pups were raised with a lactating mother until 4 weeks of age; thereafter they lived in cages with three or four animals. All were fed commercial rat chow, containing 18.7% protein, 4.7% fat and 63% carbohydrates with sufficient supply of vitamins and minerals (B&K Universal) and had tap water freely available. The study was approved by the Animal Ethics Committee of Göteborg University, Sweden.

Dams and litters

On gestational days 8, 10 and 12 dams ($n=6$) received i.p. injections of 3.5 mg/kg human recombinant leptin, dissolved in 0.5 ml of 0.9% sterile saline and provided by Eli Lilly and Company (Lilly Corporate Center, Indianapolis, IN, USA). Control dams ($n=7$) received 0.9% sterile

saline only. In a pilot study, the above dose was shown not to lead to any fetal abortion or malformation. Multiple injections, on every second day, were used to cover the sensitive period during the second trimester when brain development is most pronounced in the fetus (Paxinos *et al.* 1991). The body weight and food intake of the dams were recorded throughout the pregnancy. Tail blood was collected from the dams on days 11, 14, 17 and 21 of pregnancy to determine if the endogenous rat leptin was influenced by injection of human recombinant leptin. Gestation lasted for 22 days. Two leptin-treated dams and one control dam did not deliver any pups. After birth, litters were counted, weighed and reduced to seven or eight pups per dam to guarantee similar feeding conditions. Each litter was also adjusted to contain approximately the same ratio of male/female pups. Pups were left undisturbed until weaning at 4 weeks of age. The offspring (males, leptin $n=11$, control $n=12$; females, leptin $n=9$, control $n=10$) were weighed regularly from 4 weeks of age. Crown–rump length was measured at 10–12 weeks of age with a Vernier caliper by the same person (Electronic Digital Caliper 0–150 mm, 0.01 mm accuracy, 30–4259; Clas Ohlsson, Göteborg, Sweden).

Food intake

On two different occasions, when the rats were 5 and 9 weeks of age, their food consumption was registered once a day (males, leptin, $n=3$ cages, controls, $n=3$ cages; females, leptin, $n=3$ cages, controls, $n=3$ cages). All were presented with the same amount of food and their intake was measured the following day by subtracting the remaining uneaten food. This was done for 1 week and calculated as food intake in g/rat per day.

Vaginal smear

A vaginal smear was obtained daily for ten consecutive days when the female rats were 8–9 weeks old to determine the estrous cycle (Smith *et al.* 1975). In this study, all rats exhibited normal cyclicity, with clear ovulation (measured as a characteristic rich amount of epithelial cells without leukocytes in the smears). Blood samples were taken the day after estrus for determination of sex hormones.

Baseline hormone levels

Blood was collected from a nick in the tail for determination of baseline hormone levels. At 5 weeks of age blood was taken (non-fasting) for analysis of serum leptin, corticosterone and insulin-like growth factor-I (IGF-I). At 6–10 weeks of age, blood was taken after an overnight fast

for determination of leptin, testosterone, 17β -estradiol and progesterone. At 11 weeks of age, blood was collected (non-fasting) for serum osteocalcin analysis.

Dual energy X-ray absorptiometry (DXA)

Bone mineral content (BMC) and areal bone mineral density (BMD) (BMC/cm^2) were measured with the DEXA Sabre and Sabre Research software (both from Norland Medical Systems, Inc., Fort Atkinson, WI, USA).

Ex vivo measurements of the left tibia and vertebra L_6 were performed on excised bones placed on a 1 cm thick Plexiglass table. All bones compared were measured in the same scan (Windahl *et al.* 1999).

Peripheral quantitative computerized tomography

Computerized tomography was performed with a Stratec peripheral quantitative computerized tomography (pQCT) XCT Research M (software version 5.4B; Norland Medical Systems) operating at a resolution of 0.07 mm.

Mid-diaphyseal pQCT scans of the left tibia were performed to determine cortical volumetric BMD, the cortical cross-sectional area, the periosteal circumference, endosteal circumference and cortical thickness. In rats, the mid-diaphyseal region contains mostly cortical bone (Windahl *et al.* 1999).

Metaphyseal pQCT scans of the left tibia were performed to measure trabecular volumetric BMD. The scan was positioned in the metaphysis at a distance from the distal growth plate corresponding to 5% of the total length of the tibia (an area containing cortical and trabecular bone). The trabecular bone was defined by setting an inner threshold to 40% of the total area. The interassay coefficient of variation was <2%. It should be emphasized that DXA provides the areal BMD, whereas the pQCT gives the real/volumetric BMD. Thus DXA gives the mineral content per area, not per volume. Therefore, a factor regulating the outer dimensions of a bone will affect the areal BMD (DXA) but not the volumetric BMD (pQCT).

Tissues

The rats were killed with an i.v. injection of KCl. The brain was quickly removed and the hypothalamus was dissected, snap frozen in liquid nitrogen and stored at -80°C . The gonads and the muscles of the hind limb (extensor digitorum longus, soleus, tibialis anterior) were rapidly excised and weighed. Furthermore, epididymal/parametrial, mesenteric, retroperitoneal and inguinal adipose tissues were dissected out by the same person and weighed. The left femur and tibia, and vertebra L_6 were

excised for length measurement with the Vernier caliper, also by the same person, and analyses of tibia and vertebra L_6 in DXA and tibia in pQCT.

Preparation of protein extracts

The frozen hypothalamus from each animal was placed into an ultracentrifuge Eppendorf tube containing five volumes of ice-cold buffer (20 mM Tris, 1 mM EDTA, 10 mM sodium molybdate, 10% glycerol and 1 mM dithiothreitol) with protease inhibitors. Tissue and cells were disrupted with a Soniprep 150 Ultrasonic disintegrator (MSE Scientific Instruments, Sussex, UK). Complete homogenization was confirmed by light microscopy. Following centrifugation at 2°C for 45 min at 105 000 g (Optima TLX Ultracentrifuge, Beckman Coulter, Inc., Palo Alto, CA, USA), the supernatant was collected, aliquoted and stored at -80°C . Protein content was determined using the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL, USA).

Western blot analysis of ObR protein

A sample from each individual (25 μg total protein from the hypothalamus) was mixed with $4\times$ SDS sample buffer, boiled for 5 min and then resolved by electrophoresis in 8% SDS-PAGE gels in Tris-glycine-SDS buffer. Protein was electrophoretically transferred to polyvinylidene difluoride Western blotting membranes (Roche Molecular Biochemicals, Mannheim, Germany) in Tris-glycine-methanol buffer (overnight at 4°C , 200 mA) using a Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were split in halves for detection of ObR or actin. The membrane was blocked with 5% BSA (ObR) or 5% non-fat dry milk (actin) in Tris-buffered saline-Tween (TBS-T) at 4°C and then washed briefly in TBS-T. For detection of actin, the membrane was incubated for 1 h at room temperature in TBS-T with 1% non-fat dry milk containing the appropriate dilution of antibody (1:5000 monoclonal anti-actin (clone AC-40; Sigma, St Louis, MO, USA)). For detection of ObR, the membrane was incubated overnight at 4°C in TBS-T with 1% BSA containing the appropriate dilution of antibody (1:1000 goat polyclonal anti-ObR (Research Diagnostics, Flanders, NJ, USA)). This antibody detects the amino-terminal part of the ObR, common for all splice variants. Membranes were washed in TBS-T and placed for 1 h at room temperature in TBS-T containing the peroxidase-conjugated secondary antibody at appropriate dilution, with 1% non-fat dry milk (anti-mouse, dilution 1:5000 (Amersham Life Science, Arlington Heights, IL, USA)) or 1% BSA (anti-goat, dilution 1:10 000 (sc-2020; Santa Cruz Biotechnology, Santa Cruz, CA, USA)). Blots were washed in TBS-T and visualized with Western blot Chemi-luminescence Reagent Plus

(NEN Life Science Products, Boston, MA, USA), exposed to ECL hyperfilm (Amersham Life Science) and quantified on the Fluor-S MultiImager using the Quantity One software (Bio-Rad).

Analytical methods

Blood was collected in heparinized microtubes and centrifuged immediately in a microcentrifuge. Testosterone was measured with a solid-phase RIA (Coat-A-Count Total Testosterone; Diagnostic Products, Los Angeles, CA, USA) while 17β -estradiol was determined with an Ultra-sensitive Estradiol RIA (Diagnostic Systems Laboratories, Webster, TX, USA). Progesterone was assayed with a commercially available enzyme immunoassay (progesterone ELISA; Biomar Diagnostic Systems, Marburg, Germany). Corticosterone was determined with a RIA (RSL ^{125}I -corticosterone RIA; ICN Biomedicals, Costa Mesa, CA, USA) and similarly for rat leptin in dams. The cross-reactivity with human leptin was $<2\%$ (Rat leptin RIA kit; Linco Research, St Louis, MO, USA). Rat leptin in offspring was assayed with a commercially available enzyme immunoassay, which cross-reacts to 90% with rat leptin (Mouse Leptin ELISA; Crystal Chem., Inc., Chicago, IL, USA). Serum IGF-I was measured by double-antibody IGF-binding protein-blocked RIA (Blum & Breier 1994).

Serum osteocalcin levels were measured by an enzyme-linked immunosorbent assay (Rat-MID osteocalcin ELISA; Osteometer BioTech A/S, Herlev, Denmark).

Statistical analysis

All results are presented as the means $\pm$ S.E. The statistical method used was the Student's *t*-test, from the Stat-View program in the Macintosh system. For statistical analysis of body weight development, repeated measures ANOVA (time $\times$ treatment) was performed. Where appropriate, for comparisons between groups at different time points Student's *t*-test was used. $P < 0.05$ was considered significant.

Results

Dams and litters

The body weight of the dams increased throughout pregnancy, but there were no significant differences in body weight between the two groups at any time point (data not shown). Nor were there any significant differences in endogenous maternal leptin levels on days 11, 14, 17 and 21 of pregnancy between dams injected with human recombinant leptin or vehicle (data not shown). A significant reduction in food intake of 16% ($P < 0.05$) was observed in leptin-exposed dams the day after the first injection. Food intake was thereafter not significantly

changed. At birth, no significant differences were found in the total number of progeny per dam (leptin 8.0 ± 2.0 and control 7.2 ± 0.9 pups per dam) or the ratio of male births to total births in each litter (leptin 0.51 ± 0.05 and control 0.48 ± 0.06) between leptin-injected dams and control dams.

Body weight development and body composition

Male offspring of leptin-treated dams had significantly lower birth weights than male offspring of control dams (6.2 ± 0.1 and 6.7 ± 0.1 g respectively, $P < 0.01$), but the difference had disappeared at 1 week of age. There was no significant difference in birth weight for female leptin-exposed offspring and control offspring (6.1 ± 0.1 and 6.4 ± 0.1 g respectively). Table 1 shows the body weight development in male and female offspring from 1 to 10 weeks of age. Female leptin-exposed offspring weighed significantly less than control offspring from 8 up to 10 weeks of age when comparing individual time points with Student's *t*-test and when performing a repeated-measures ANOVA there was a significant interaction between time and treatment on the body weight of females. For male offspring there were no significant differences between the groups. Food intake did not differ between the groups at 5 and 9 weeks, for either female or male offspring.

Male leptin offspring had significantly reduced epididymal ($P < 0.05$) and retroperitoneal ($P < 0.01$) adipose tissue weights in relation to total body weight, as compared with controls (Table 2). A significant enlargement of testicles ($P < 0.05$) was also present in these male offspring. Significantly reduced weights of parametrial ($P < 0.05$) and retroperitoneal ($P < 0.05$) adipose tissues in relation to total body weight were found in female leptin offspring, together with significantly heavier soleus muscle ($P < 0.05$) (Table 2).

As shown in Table 3, the crown-rump and tibia lengths and the height of vertebra L_6 were significantly decreased in male leptin-exposed offspring, whereas the difference in femur length did not reach statistical significance. In female offspring, as shown in Table 3, the lengths of tibia and femur were significantly decreased, but the crown-rump length and the height of vertebra L_6 were not different from the control group.

Bone mineral status as determined by DXA

Male leptin-exposed offspring demonstrated decreased BMC and areal BMD in both the tibia and vertebra L_6 (Table 4). In female offspring no differences were seen in DXA measurements of the tibia and vertebra L_6 between leptin-exposed and control offspring (Table 4).

Trabecular BMD

The pQCT technique was used to measure the trabecular volumetric BMD in the metaphysis of the tibia. Neither

Table 1 Development of total body weight (g) from 1 to 10 weeks of age in control and leptin-exposed male and female offspring

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
Males										
Leptin (<i>n</i> =11)	18.5 ± 0.2	35.6 ± 0.4	52.0 ± 0.9	87.1 ± 1.8	118 ± 2	155 ± 3	195 ± 4	241 ± 5	266 ± 6	287 ± 6
Controls (<i>n</i> =12)	17.9 ± 0.5	35.5 ± 1.0	52.4 ± 1.4	88.5 ± 2.3	122 ± 3	161 ± 4	200 ± 6	248 ± 8	272 ± 7	298 ± 9
Females										
Leptin (<i>n</i> =9)	17.3 ± 0.2	35.1 ± 0.5	51.0 ± 1.1	81.5 ± 1.4	105 ± 2	125 ± 2	147 ± 2	166 ± 3*	176 ± 4*	189 ± 3*
Control (<i>n</i> =10)	17.7 ± 0.6	34.6 ± 1.0	50.3 ± 1.6	82.5 ± 2.3	109 ± 2	128 ± 4	157 ± 5	178 ± 5	190 ± 6	204 ± 5

Data are means ± SE, $P < 0.001$ for time in both genders and for time × treatment in females (ANOVA repeated measures).
 * $P < 0.05$ for individual time points (Student's *t*-test).

male nor female offspring showed any statistically significant differences between the groups in trabecular volumetric BMD (data not shown).

Cortical bone parameters

Cortical bone parameters were studied in detail in mid-diaphyseal pQCT scans of the tibia. The results for tibiae in male offspring are shown in Table 5. Cortical BMC was decreased in leptin-exposed offspring as compared with control, and this decrease was caused by a reduction in the cortical cross-sectional area whereas cortical BMD was unchanged. The reduction in the cortical cross-sectional area was associated with an unchanged endosteal circumference and decreased periosteal circumference, and consequently a decrease in cortical thickness.

Cortical volumetric density of the tibia was increased in leptin-exposed female offspring, whereas a significantly reduced cortical cross-sectional area still resulted in a decreased cortical BMC (Table 5). The reduction in the cortical cross-sectional area was associated with a decrease in both the periosteal and endosteal circumferences and an unchanged cortical thickness.

Hormones

In male offspring, plasma concentrations of sex steroids in leptin-exposed and control offspring at 9 weeks of age were as follows: testosterone 3.80 ± 1.45 nmol/l, $n=11$, and 1.57 ± 0.55 nmol/l, $n=12$; and progesterone 2.35 ± 0.60 nmol/l, $n=11$, and 3.45 ± 0.85 nmol/l, $n=12$. 17β -Estradiol was below detectable levels. The plasma concentrations of corticosterone, IGF-I and osteocalcin did not show any significant differences between leptin-exposed and control offspring (data not shown). Circulating leptin levels measured at 5, 6, 8 and 9 weeks of age did not differ between the groups either (data not shown).

In female offspring, plasma concentrations of testosterone (0.136 ± 0.01 nmol/l, $n=9$, and 0.106 ± 0.01 nmol/l, $n=10$, $P < 0.05$) were significantly higher in leptin-exposed offspring, whereas plasma concentrations of progesterone (35.7 ± 10.1 nmol/l, $n=9$, and 25.2 ± 3.7 nmol/l, $n=10$) and 17β -estradiol (17.7 ± 2.1 pmol/l, $n=9$, and 15.8 ± 1.9 pmol/l, $n=10$) did not differ. Plasma levels of osteocalcin (168 ± 6.7 ng/ml, $n=8$, and 222 ± 18 ng/ml, $n=10$, $P < 0.05$) were significantly lower in the leptin-exposed offspring whereas corticosterone and IGF-I were unaffected (data not shown). Determination of circulating leptin at 5, 6, 8 and 9 weeks of age, did not reveal any differences between the groups (data not shown).

ObR expression in the hypothalamus

There were no differences in hypothalamic ObR protein levels expressed as the ObR:actin ratio, either in the

Table 2 Weights of extensor digitorum longus (EDL), soleus (SOL) and tibialis anterior (TIB) muscles, epididymal/parametrial, retroperitoneal, mesenteric and inguinal adipose tissues and gonads (g/kg body weight) in 10-week-old male and 12-week-old female leptin-exposed and control offspring

	Muscles			Adipose tissue				Testicles/ ovaries
	EDL	SOL	TIB	Epididymal/ parametrial	Retroperitoneal	Mesenteric	Inguinal	
Males								
Leptin (<i>n</i> =10)	0.38 ± 0.01	0.37 ± 0.02	1.75 ± 0.03	17.1 ± 0.7*	11.5 ± 1.2**	9.5 ± 0.4	13.0 ± 1.0	11.0 ± 0.2*
Control (<i>n</i> =10)	0.36 ± 0.01	0.35 ± 0.02	1.69 ± 0.04	19.6 ± 0.7	16.3 ± 0.9	10.9 ± 0.5	14.0 ± 1.2	10.2 ± 0.3
Females								
Leptin (<i>n</i> =8)	0.38 ± 0.01	0.44 ± 0.03*	1.75 ± 0.06	19.2 ± 1.4*	10.3 ± 0.9*	10.2 ± 0.4	11.9 ± 0.7	0.44 ± 0.01
Control (<i>n</i> =10)	0.37 ± 0.01	0.36 ± 0.01	1.85 ± 0.04	26.0 ± 2.4	14.5 ± 1.2	11.4 ± 0.9	12.6 ± 0.9	0.40 ± 0.02

Data are means ± S.E. (Student's *t*-test), *P<0.05, **P<0.01 compared with control.

male offspring (0.469 ± 0.063 in leptin rats, *n*=9, and 0.528 ± 0.044 in controls, *n*=9) or in the female offspring (0.413 ± 0.039 in leptin rats, *n*=8, and 0.362 ± 0.037 in controls, *n*=9).

Discussion

The present study reports that maternally administered human recombinant leptin leads to lean male and female offspring with reduced skeletal growth. Obesity and intake of diets rich in polyunsaturated fatty acids are conditions

associated with hyperleptinemia that can prevail during human pregnancy (Cha & Jones 1998, Levine *et al.* 1999).

In the adult, as well as in the suckling and juvenile rat, leptin is an anti-obesity adipostatic hormone reflecting body fat content and regulating food intake and energy metabolism (Zhang *et al.* 1994, Campfield *et al.* 1995, Rayner *et al.* 1997, Yuan *et al.* 2000). Furthermore, it coordinates the neuroendocrine response to starvation (Ahima *et al.* 1996). In younger rats though it is suggested that leptin acts to facilitate the onset of puberty, and in humans leptin levels increase before entering puberty (Cheung *et al.* 1997, Mantzoros *et al.* 1997). Ahima *et al.*

Table 3 Length of tibia, femur and crown–rump and height of vertebra L₆ (mm) in 10-week-old male and 12-week-old female leptin-exposed and control offspring

	Crown–rump	Tibia	Femur	Vertebra L ₆
Males				
Leptin (n=10)	232 ± 1.6*	37.1 ± 0.2**	31.5 ± 0.2	7.05 ± 0.10**
Control (n=10)	237 ± 1.0	37.9 ± 0.2	31.7 ± 0.1	7.40 ± 0.07
Females				
Leptin (n=8)	209 ± 2.5	34.6 ± 0.1**	29.1 ± 0.1**	6.84 ± 0.09
Control (n=10)	211 ± 1.8	35.2 ± 0.2	29.9 ± 0.2	6.93 ± 0.11

Data are means ± S.E. (Student's *t*-test), *P<0.05, **P<0.01 compared with control.

Table 4 BMC and areal BMD of tibia and vertebra L₆ as measured with DXA in 10-week-old male and 12-week-old female leptin-exposed and control offspring

	Tibia		Vertebra L ₆	
	BMD (g/cm ²)	BMC (g)	BMD (g/cm ²)	BMC (g)
Males				
Leptin (n=10)	0.150 ± 0.001*	0.226 ± 0.002**	0.163 ± 0.002**	0.081 ± 0.001***
Control (n=10)	0.155 ± 0.001	0.239 ± 0.003	0.175 ± 0.003	0.091 ± 0.002
Females				
Leptin (n=8)	0.154 ± 0.002	0.212 ± 0.004	0.159 ± 0.002	0.069 ± 0.002
Control (n=10)	0.156 ± 0.002	0.223 ± 0.004	0.166 ± 0.003	0.076 ± 0.003

Data are means ± S.E. (Student's *t*-test), *P<0.05, **P<0.01 compared with control.

Table 5 Cortical bone parameters of tibia as measured with pQCT in 10-week-old male and 12-week-old female leptin-exposed and control offspring

	Cortical BMD (mg/mm ³)	Cortical cross-sectional area (mm ²)	Cortical BMC (mg/mm)	Cortical thickness (mm)	Cortical periosteal circumference (mm)	Cortical endosteal circumference (mm)
Males						
Leptin (n=10)	1.23 ± 0.00	3.84 ± 0.04***	4.72 ± 0.05***	0.505 ± 0.008*	9.20 ± 0.05**	6.02 ± 0.05
Control (n=10)	1.23 ± 0.01	4.12 ± 0.05	5.08 ± 0.06	0.532 ± 0.006	9.42 ± 0.05	6.08 ± 0.06
Females						
Leptin (n=8)	1.30 ± 0.00**	3.27 ± 0.07**	4.23 ± 0.09*	0.485 ± 0.011	8.27 ± 0.07**	5.22 ± 0.10*
Control (n=10)	1.27 ± 0.01	3.56 ± 0.06	4.52 ± 0.07	0.501 ± 0.007	8.68 ± 0.07	5.53 ± 0.07

Data are means ± S.E. (Student's *t*-test), *P<0.05, **P<0.01, ***P<0.001 compared with control.

(1998) showed that a discrete leptin surge exists in mice during the second postnatal week which is independent of fat mass or food intake. It is suggested that, during the early postnatal period, leptin is important for the maturation and function of neuroendocrine axes, and this is independent of its later role as a regulator of energy stores (Ahima *et al.* 1998, Mistry *et al.* 1999). Growing evidence supports the hypothesis that leptin acts as an important factor for prenatal growth and development. In both humans and rats, circulating plasma leptin levels are elevated approximately 2-fold during pregnancy, in contrast to the mouse where plasma levels of leptin are elevated 30- to 40-fold during pregnancy (Tomimatsu *et al.* 1997, Helland *et al.* 1998, Herrera *et al.* 2000). Also newborns display high leptin levels (Helland *et al.* 1998, Herrera *et al.* 2000). The high levels of leptin during pregnancy are not contributed from the increase in adipose tissue alone, since leptin is expressed in the placenta and in the fetus as well. This implies that leptin may have important functions perinatally.

There is a relationship between body fat and bone mass. *ob/ob* mice, lacking leptin, have a phenotype with obesity and high bone mass (Felson *et al.* 1993, Ducy *et al.* 2000). The prenatally leptin-exposed animals presented in this study were lean and their skeletal growth was reduced. The general leanness was not due to decreased food intake in leptin-exposed rats. The reduction in adipose tissue seemed to be accompanied by a generalized increase in muscle weight per body weight, which could be explained by the increased testosterone levels, since testosterone is well known to exert anabolic effects on muscle (Holmang *et al.* 1990). It is suggested that leptin plays a role in fetal bone metabolism (Oguchi *et al.* 2000). In both male and female offspring, cortical bone dimensions were reduced, but there was no effect on cancellous bone. The length of tibiae was affected, as was the circumference, leading to reduction in cortical BMC. In female offspring these changes were accompanied by reduced circulating levels of osteocalcin, an important marker for bone formation.

The placenta and several tissues in the fetus express ObR (Hoggard *et al.* 1997). The presence of ObR in the

placenta suggests a paracrine role for leptin in this tissue, leading to production of other hormones in the placenta which are vital for pregnancy and growth (Henson & Castracane 2000). Whether leptin is able to pass the placental barrier or not is unconfirmed. A recent study demonstrated that a human recombinant immunoadhesin leptin fusion protein was not able to pass the placenta (Yamashita *et al.* 2001). However, since the placenta is not an absolute barrier it is possible that there is some passage of leptin from the mother to the fetus (Chandorkar *et al.* 1999). It is not clear whether the findings in this study are the result of a direct effect of leptin on the fetus or an indirect effect of leptin binding to ObR in the placenta and thereby influencing placental/fetal hormone production or substrate availability (Yamashita *et al.* 2001). Hyperleptinemia in the pregnant dam may also lead to secondary hormonal reactions in the dam, producing hormones that in turn pass the placenta, affect the fetus and cause the programming seen in this study. The leptin exposure did lead to a 16% reduction in food intake of the dam on the first injection occasion but food intake did not differ from controls thereafter. This may have influenced the nutrient availability for the fetus. However, since the magnitude of the decrease in food intake was very moderate and did not last for more than 24 h it is unlikely to have exerted any major effects.

Leptin exerts direct effects on skeletal muscle and adipocytes *in vitro* and hyperleptinemia has been shown to result in body fat depletion through extrahypothalamic actions *in vivo* (Siegrist-Kaiser *et al.* 1997, Ceddia *et al.* 1998, Wang *et al.* 1999). ObR is present in adipose tissue, skeletal muscle and human and rat primary osteoblasts and bone samples (Hoggard *et al.* 1997, Evans *et al.* 2001). In the 14-day-old mouse fetus, ObR was shown to be expressed in cartilage/bone tissue (Hoggard *et al.* 1997). It is therefore possible that the effects on cortical bone formation and body composition seen in the prenatally leptin-exposed offspring of this study were mediated through peripheral effects of leptin during development. ObR number, receptor sensitivity or intracellular signal transduction may have been

changed in the affected peripheral tissue. These effects may occur during a defined time window of development and persist into adulthood.

There is also evidence suggesting primarily central effects. In adult rats, leptin regulates appetite and energy expenditure by interacting with the long isoform of the ObR expressed in the arcuate, paraventricular and supraoptic nuclei of the hypothalamus (Campfield *et al.* 1995, Shioda *et al.* 1998). It has also been suggested that leptin can regulate bone formation through central mechanisms (Ducy *et al.* 2000). *ob/ob* mice, lacking leptin, have a phenotype with high trabecular bone mass. Intracerebroventricular leptin administration in these mice and in wild-type mice led to a substantial reduction of their bone mass and it was therefore suggested that the effect of leptin on bone mass is mediated through a central mechanism and not only through autocrine or paracrine mechanisms (Ducy *et al.* 2000). Recently, *fa/fa* rats, lacking a functional ObR, have also been shown to have an increase in both trabecular and cortical bone masses, suggesting that ObR is involved in leptin regulation of bone mass (Schilling *et al.* 2001).

In this study, the fetus was exposed to hyperleptinemia during the second trimester, a period of extensive brain development. It is therefore possible that the prenatal leptin exposure led to permanent programming of the fetal central nervous system, leading to altered regulations of body composition, bone formation and neuroendocrine axes in the adult offspring, all factors known to be regulated centrally. Previous studies have suggested that leptin influences brain development at critical stages (Bereiter & Jeanrenaud 1979, Ahima *et al.* 1999). No hypothalamic expression of ObR could be detected in either mouse or rat fetuses on day 14 of gestation, but ObRs were shown to be expressed in other parts of the brain (Hoggard *et al.* 1997, Matsuda *et al.* 1999). In this study, no significant changes in hypothalamic ObR protein expression was found between leptin-exposed and control offspring. However, protein for examination of ObR expression was extracted from whole hypothalamus, and differences in specific hypothalamic nuclei cannot be ruled out. Nor do we know anything about the function of the ObRs after perinatal hyperleptinemia. It is also possible that ObR expression and function in other parts of the brain are affected by high leptin levels during early fetal development, leading to the effects seen in this study.

In summary, our results show for the first time that hyperleptinemia early in pregnancy can permanently program the offspring to a phenotype with reduced skeletal growth and reduced adipose tissue at adult age. This implies that leptin, among its other functions, is also an important mediator in the fetal development of bone and adipose tissue. Further studies are required to elucidate the underlying mechanisms of this programming.

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