Affected skeletal growth but normal bone mineralization in rat offspring after prenatal dexamethasone exposure

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Abstract

Events occurring early in life or prenatally are able to play important roles in the pathogenesis of diseases in adult life. Different sorts of stress or hormonal influences, during particular periods of pregnancy, may result in persisting or transient changes in physiology. Glucocorticoids are used for the treatment of a variety of diseases, to promote organ maturation and to prevent preterm delivery. Glucocorticoids are also known to affect skeletal growth and adult bone metabolism. The aim of the present study was to investigate whether exposure to dexamethasone (Dex) during fetal life has any effect on skeletal growth and/or bone mineral density in adult rat offspring.

Pregnant rats were given injections of either Dex (100µg/kg) or vehicle on days 9, 11 and 13 of gestation. Dex-exposed male but not female rat offspring showed transient increases in crown–rump length and tibia and femur lengths at 3–6 weeks of age. In contrast, the cortical bone dimensions were altered in 12-week-old female but not male Dex-exposed offspring. The areal bone mineral densities of the long bones and the spine, as determined by dual X-ray absorptiometry, and trabecular as well as cortical volumetric bone mineral density, as measured using peripheral quantitative computerized tomography, were unchanged in both male and female Dex-exposed offspring.

In conclusion, prenatal Dex exposure affects skeletal growth in a gender-specific manner, while the mineralization of bones is unaffected in both male and female offspring.

Introduction

It has been shown that events occurring early in life or prenatally are able to play important roles in the pathogenesis of adult disease in both humans and animals (Barker et al. 1993, Benediktsson et al. 1993). The process whereby a stimulus or insult at a sensitive or critical period of development has long-term effects is termed ‘programming’ (Lucas 1991). Dexamethasone (Dex) is a potent synthetic glucocorticoid lacking mineralocorticoid activity, and it passes through the placenta without being inactivated by the enzyme 11β-hydroxysteroid dehydrogenase. Exposure to Dex late in pregnancy has been claimed to result in a reduced birth weight, and in hyperinsulinæmia and hypertension in adulthood (Benediktsson et al. 1993, NIH 1995). A recent clinical study suggests that low birth weight is associated with insulin resistance in children and adolescents (Ornoy & Altshuler 1976, Hofman et al. 1997, Robinson et al. 2000). A recent study has shown that prenatal exposure to Dex leads to increased fat depots in the adult rat. One of the possible mechanisms involved is programming of the fetal hypothalamo–pituitary–adrenal (HPA) axis (Matthews 2000). The HPA axis, which is central to the integration of the individual’s endocrine and behavioural response to stress, appears to be highly sensitive to an excess of glucocorticoids during development (Clark 1998).

Glucocorticoids are widely used to treat inflammatory diseases, asthma, and as immunosuppressives. High endogenous levels of cortisol are seen during infections and stressful events. Furthermore, 10% of pregnant women in North America and Europe are treated with synthetic glucocorticoids between weeks 24 and 34 of gestation to promote fetal lung maturation in fetuses at risk of being delivered prematurely (Matthews 2000). Long-term
therapy with high doses of glucocorticoids has been shown to suppress skeletal growth in childhood (Blodgett et al. 1956). Possible mechanisms for this might include a central mechanism through the somatotrophic hormone axis and direct effects on the growth plate by suppression of chondrocyte proliferation (Klaus et al. 2000).

Glucocorticoids also have profound effects on adult bone metabolism, and prolonged exposure to excessive amounts of glucocorticoids results in osteoporosis (Cushing 1932). However, the exact mechanism for this effect remains to be elucidated. Fetal rat osteoblasts express receptors for glucocorticoids (Chen & Feldman 1979) and a direct effect on bone formation is therefore possible. High levels of glucocorticoids inhibit bone formation via complex direct and indirect mechanisms, including inhibition of osteoblastic cell replication, decreased collagen expression and increased collagenase expression (Canalis 1996). Furthermore, high levels of glucocorticoids also increase bone resorption (Hofbauer et al. 1999). Increasing evidence suggests that a mechanism for glucocorticoid-induced bone diseases arises from changes in osteoblast and osteocyte numbers due to apoptosis of these cells (Hughes & Boyce 1997, Weinstein et al. 1998, Weinstein & Manolagas 2000).

The aim of the present study was to investigate whether glucocorticoids can cause long-lasting effects on skeletal growth and/or mineralization of bones in rat offspring after early prenatal exposure.

**Materials and Methods**

**Animals**

Timed-pregnant nulliparous Wistar rats (B&K Universal, Sollentuna, Sweden) were housed one animal per cage until parturition, under controlled conditions, at a temperature of 21 ± 1 °C, humidity of 55–65% and lights on from 0700 to 1900 h. They received a commercial rat chow (containing 18.7% protein, 4.7% fat and 63% carbohydrates, with a sufficient supply of vitamins and minerals) (B&K Universal). Tap water was available *ad libitum*. Pups were raised with a lactating mother until 4 days of age. Six offspring of each gender and group were killed by an overdose of anaesthesia at 6 weeks of age. The remaining offspring were kept until 10 weeks of age for male rats and 12 weeks of age for female rats. The gonads and the muscles of the hind limb (extensor digitorum longus, tibialis anterior and soleus muscles) were rapidly excised and weighed. Left femur, tibia and vertebra L6 were excised and stored in 70% ethanol until analysis.

**Body and tissue weights**

Total body weight and the weight of muscles and gonads were measured on a balance (PG 3001-S; Mettler Toledo, Greifensee, Switzerland). The body length, left femur, left tibia and the height of vertebra L6 were measured by the same person with a digital vernier calliper (Electronic Digital Calliper 0–150 mm, 0.01 mm accuracy, 30–4259; Clas Ohlsson AB, Insjön, Sweden).

**Food intake**

At 8–10 weeks of age, food consumption for each cage (three to four animals per cage) was recorded every second day for 20 days and calculated as food intake in grams per rat per day. All animals were presented with the same amount of food and their intake was measured the following day by subtracting the uneaten food plus spillage from the total food given (male cages: dex n = 3, controls n = 2; female cages: dex n = 3, controls n = 3).

**Baseline hormone levels**

At 5–10 weeks of age, blood samples were collected from a nick in the tail after the rats had been fasted overnight. Samples were taken between 0800 and 1000 h on the same day to determine levels of leptin (week 8) and testosterone, 17β-estradiol and progesterone (weeks 9–10 in females, the day after oestrus, when these hormones are known to be low compared with the pro-oestrous phase). Vaginal smears were obtained daily between 8 and 10
weeks of age, to determine the oestrous cycle. Corticosterone was analysed in the non-fasting state at 5 weeks of age (Smith et al. 1975).

**Hormone analysis**

Blood was collected in heparinized microtubes and centrifuged immediately in a microcentrifuge at 4 °C. Testosterone was measured with a solid-phase RIA (Coat-A-Count Total Testosterone; Diagnostic Products, Los Angeles, CA, USA); intra-assay coefficient of variation (CV) <10%, interassay CV <12% and detection level above 0.14 nmol/l. Progesterone was assayed with a commercially available ELISA (Biomark Diagnostic Systems, Marburg, Germany); intra-assay CV was <9%, interassay CV was <10% and detection level was above 0.6 nmol/l. 17β-Oestradiol was measured with an ultra-sensitive oestradiol RIA (Diagnostic Systems); intra-assay CV was <9%, interassay CV was <12% and detection level was above 0.1 pmol/l. Corticosterone was determined by RIA (RSL 125I corticosterone RIA; ICN Biomedicals, Costa Mesa, CA, USA); intra-assay CV was <10%, interassay CV was <10% and detection level was above 25 ng/ml. Leptin was determined by RIA (Rat Leptin RIA kit; Linco Research, St Louis, MO, USA); intra-assay CV was <5%, interassay CV was <6% and detection level was above 0.5 ng/ml. Serum insulin-like growth factor I (IGF-I) was measured by a double-antibody IGF-binding protein–blocked RIA (IGF-I, IGFBP blocked; Mediagnost, Tubingen, Germany); interassay CV was <8%, cross-reactivity with IGF-II was less than 0.05% and detection level was above 0.05 mg/ml. Serum osteocalcin levels were measured by an ELISA (Rat-MID osteocalcin ELISA; Osteometer BioTech A/S, Herlev, Denmark); intra-assay CV was <8%, interassay CV was <10% and detection level was above 62 ng/ml. Bone mineral content (BMC) and areal bone mineral density (BMD; BMC/cm²) were measured using pDEXA (Smith et al. 1975).

An average of 60 growth plate measurements (six sections, ten measurements/section) was calculated for each tibia and femur. For dual X-ray absorptiometry (DXA) measurements, the interassay CV was less than 4%.

**Peripheral quantitative computerized tomography (pQCT)**

Computerized tomography was performed with the Stratec pQCT XCT Research M (software version 5.4B; Norland Medical Systems Inc.) operating at a resolution of 70 µm.

Mid-diaphyseal pQCT scans of femur and tibia were performed to measure the cortical volumetric BMD, the cortical cross-sectional area, the cortical thickness and the periosteal and endosteal circumferences. The mid-diaphyseal region of the femur and tibia in rats contains mostly cortical bone (Windahl et al. 1999).

Metaphyseal pQCT scans of left femur and 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 7% of the total length of the femur and 5% of the total length of the tibia (an area containing a central portion of trabecular bone). The trabecular bone region was defined by setting an inner threshold at 45% of the total area. The interassay CV values for the pQCT measurements were less than 2%.

It should be emphasized that the DXA technique gives 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).

**Results**

**Dams and litters**

The body weight of the dams increased throughout pregnancy and there was no significant difference in body weight between the two groups at any time-point (data not shown). To confirm that the Dex treatment was functioning, Dex-injected dams were analysed for serum corticosterone. This revealed suppressed serum corticosterone levels 4 h after injection, compared with basal levels and those in control dams (before first injection: Dex, 639 ± 86 ng/ml; controls, 503 ± 80 ng/ml; after second injection: Dex, 50 ± 20 ng/ml; controls, 371 ± 73 ng/ml; P<0.01). Litter size for the Dex-exposed group was lower compared with the control group (Dex, 8.6 ± 0.58; controls, 12.0 ± 0.58; P<0.05).

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**Statistical methods**

Distribution of variables are given as means ± S.E.M. Student’s t-test was used for comparison between the treatment groups and the control groups and a two-tailed P value of <0.05 was considered significant.
Bone mineral status as determined by DXA and pQCT

Arents and others · Prenatal dexamethasone affects rat skeletal growth

Table 1 Development of total body weight (g) from birth to 12 weeks of age. Values are means ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>At birth</th>
<th>Week 1</th>
<th>Week 2</th>
<th>After sexing</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
<th>Week 7</th>
<th>Week 8</th>
<th>Week 9</th>
<th>Week 10</th>
<th>Week 12</th>
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</thead>
<tbody>
<tr>
<td>Control Female + male (n=30)</td>
<td>7 ± 0·1</td>
<td>16 ± 0·3</td>
<td>28 ± 0·5</td>
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<td>40 ± 0·6</td>
<td>68 ± 1·2</td>
<td>103 ± 1·6</td>
<td>136 ± 2·3</td>
<td>161 ± 3·4</td>
<td>182 ± 3·8</td>
<td>199 ± 4·3</td>
<td>218 ± 7·2</td>
<td>241 ± 9·1</td>
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<tr>
<td>Female (n=18)</td>
<td>42 ± 1·0</td>
<td>71 ± 1·4</td>
<td>115 ± 1·6</td>
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<td>163 ± 3·8</td>
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<tr>
<td>Male (n=12)</td>
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<tr>
<td>Dex-treated Female + male (n=28)</td>
<td>7 ± 0·1</td>
<td>16 ± 0·3</td>
<td>34 ± 1·0***</td>
<td></td>
<td>46 ± 0·8***</td>
<td>74 ± 1·4***</td>
<td>106 ± 2·0</td>
<td>137 ± 3·0</td>
<td>159 ± 4·1</td>
<td>176 ± 5·2</td>
<td>193 ± 5·2</td>
<td>209 ± 5·3</td>
<td>233 ± 8·3</td>
</tr>
<tr>
<td>Female (n=19)</td>
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<tr>
<td>Male (n=16)</td>
<td>51 ± 1·4***</td>
<td>83 ± 2·0***</td>
<td>124 ± 2·1**</td>
<td></td>
<td>169 ± 2·5</td>
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</tbody>
</table>

From birth to 2 weeks of age, sex was not identified and data are therefore pooled together in control or Dex-treated offspring. From week 3, Dex-treated females are compared with control females and Dex-treated males are compared with control males; **P<0·01, ***P<0·001 (t-test).
Cortical endosteal circumference (mm) 6.23
Cortical periosteal circumference (mm) 8.93
Cortical thickness (mm) 0.43
Cortical density (mg/mm³) 1.158
Cortical area (mm²) 3.26

Leptin levels were increased at 8 weeks of age in Dex-treated female offspring (Dex, 1.25 ± 0.07 ng/ml; controls, 0.95 ± 0.10 ng/ml; P < 0.05). This was associated with an increase in adipose tissue at week 12, although it did not reach statistical significance (parametrial fat: controls, 17.7 ± 1.73 g/kg body weight (b.w.); Dex, 21.5 ± 1.78 g/kg b.w.; retroperitoneal fat: controls, 12.6 ± 1.19 g/kg b.w.; Dex, 14.6 ± 1.59 g/kg b.w.). Serum levels of IGF-I, corticosterone, testosterone, progesterone, oestradiol and osteocalcin were unchanged in Dex-treated animals compared with controls in both males and females.

Serum parameters

Leptin levels were increased at 8 weeks of age in Dex-treated female offspring (Dex, 1.25 ± 0.07 ng/ml; controls, 0.95 ± 0.10 ng/ml; P < 0.05). This was associated

Organ weights and food intake

The weights of gonads, muscles and adrenals were measured at 12 weeks of age in female rats and at 10 weeks of age in male rats. These weights did not differ between Dex-treated and control rats (data not shown). Furthermore, food intake was measured between 8 and 10 weeks.

Table 2 Development of crown–rump length (mm) from birth to 5 weeks of age. Values are means ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>At birth</th>
<th>Week 1</th>
<th>Week 2</th>
<th>After sexing</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Female + male (n=30)</td>
<td>50.7 ± 0.4</td>
<td>68.5 ± 0.5</td>
<td>87.2 ± 0.7</td>
<td>Female (n=18)</td>
<td>105.1 ± 1.2</td>
<td>128.0 ± 1.0</td>
<td>144.6 ± 0.5</td>
</tr>
<tr>
<td>Dex-treated Female + male (n=35)</td>
<td>51.0 ± 0.3</td>
<td>68.6 ± 0.6</td>
<td>90.1 ± 0.6**</td>
<td>Female (n=19)</td>
<td>106.0 ± 0.5</td>
<td>127.6 ± 0.5</td>
<td>142.4 ± 0.7*</td>
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<td></td>
<td></td>
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<td></td>
<td>Male (n=12)</td>
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<td></td>
<td>Male (n=16)</td>
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</tbody>
</table>

From birth to 2 weeks of age, sex was not identified and data are therefore pooled together in Dex-treated or control offspring. Dex-treated offspring (male and female) are compared with controls (female and male) from birth to week 2; from weeks 3 to 5, Dex-treated females are compared with control females and control males are compared with Dex-treated males; *P < 0.05, **P < 0.01, ***P < 0.001 Dex vs control.

Table 3 Length of tibia, femur and height of vertebra L₆ in male rats and controls at 6 and 10 weeks of age. Values are means ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>6 Weeks of age</th>
<th>10 weeks of age</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=6)</td>
<td>Dex (n=6)</td>
</tr>
<tr>
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<tr>
<td>Vertebræ L₆ (mm)</td>
<td>5.10 ± 0.07</td>
<td>5.14 ± 0.06</td>
</tr>
<tr>
<td>Tibia (mm)</td>
<td>29.91 ± 0.22</td>
<td>30.91 ± 0.26*</td>
</tr>
<tr>
<td>Femur (mm)</td>
<td>24.91 ± 0.15</td>
<td>25.52 ± 0.22*</td>
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</table>

*p < 0.05 Dex vs control (*t-test).

Table 4 Trabecular volumetric BMD and cortical bone parameters of femur as measured using pQCT for Dex-treated and control female offspring at 6 and 10 weeks of age. Values are means ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>6 Weeks of age</th>
<th>12 Weeks of age</th>
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<tbody>
<tr>
<td></td>
<td>Control (n=6)</td>
<td>Dex (n=6)</td>
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<tr>
<td></td>
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<tr>
<td>Metaphyseal trabecular BMD (mg/mm³)</td>
<td>0.199 ± 0.022</td>
<td>0.186 ± 0.023</td>
</tr>
<tr>
<td>Cortical density (mg/mm³)</td>
<td>1.158 ± 0.006</td>
<td>1.164 ± 0.005</td>
</tr>
<tr>
<td>Cortical area (mm²)</td>
<td>3.26 ± 0.11</td>
<td>3.32 ± 0.09</td>
</tr>
<tr>
<td>Cortical content (mg/mm)</td>
<td>3.78 ± 0.14</td>
<td>3.86 ± 0.11</td>
</tr>
<tr>
<td>Cortical thickness (mm)</td>
<td>0.43 ± 0.02</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>Cortical periosteal circumference (mm)</td>
<td>8.93 ± 0.10</td>
<td>9.22 ± 0.11</td>
</tr>
<tr>
<td>Cortical endosteal circumference (mm)</td>
<td>6.23 ± 0.13</td>
<td>6.58 ± 0.11</td>
</tr>
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</table>

pQCT analyses were performed at 6 and 12 weeks of age. *P < 0.05, **P < 0.01 (*t-test).
of age; there was no difference in food intake between the Dex-treated group and the control group (data not shown).

Discussion

The programming hypothesis, whereby events early in life or during specific fetal periods have long-lasting effects later in life, is supported by human epidemiological data and animal models. These studies have confirmed that changes in the nutritional, metabolic and hormonal environment during both prenatal and postnatal periods of life may have profound effects on body composition, glucose tolerance, blood pressure and cardiovascular diseases (Lucas 1991, Benediktsson et al. 1993, NIH 1995, Dahlgren et al. 2001, Holmäng 2001). From a clinical point of view, it is important to examine the effects of glucocorticoids on different organ systems, as they are used antenatally to accelerate the maturation of fetal respiratory and cardiovascular systems when there is a risk of preterm delivery. Postnatally, glucocorticoids are used to prevent and treat respiratory distress syndrome. Also, pregnant women with a variety of diseases are treated with steroids. Currently, very little information is available on the impact of such treatment regimens. Previous studies regarding the effects of prenatal exposure to glucocorticoids have focused on metabolic or neuroendocrine effects and not on skeletal effects. This study was therefore undertaken to investigate whether prenatal exposure to glucocorticoids in rats has any effect on skeletal growth and adult bone tissue in offspring.

We demonstrate, for the first time, that prenatal Dex exposure affects skeletal growth. Dex-treated male but not female rats demonstrated a transient increase in crown-rump length and long-bone length, indicating that Dex induces gender-specific programming of skeletal growth. Gender-specific programming is supported by measurement of cortical dimensions, including periosteal and endosteal circumferences and cortical thickness; these were altered in adult female but not male Dex-treated offspring. This observed sex difference might be explained by altered numbers of glucocorticoid receptors on cells in bone. However, we believe that a more likely explanation is through a central mechanism, including changes in number of various centrally located receptors. It has previously been demonstrated that in maternal glucocorticoid treatment programmes the HPA axis functions in a sex-specific manner and that these changes are associated with modification in corticosteroid receptor expression in the adult brain (Liu et al. 2001). The areal BMD of the long bones and the spine, as well as the trabecular and volumetric BMD, were unchanged in the Dex-treated offspring. Therefore, prenatal Dex exposure affects skeletal growth but not mineralization. Direct effects of mineralization in utero do not show as well, as there is not much mineralization so early in developing pups, but our indirect findings in programming is a way in which this might be studied.

Another mechanism for glucocorticoid-induced bone diseases arises from changes in osteoblasts and osteocytes number due to apoptosis of these cells (Hughes & Boyce 1997, Weinstein et al. 1998, Weinstein & Manolagas 2000). The lack of change in bone mineralization, seen in the present study, could be because glucocorticoids increase osteoclast apoptosis in rats and thereby counterbalance the effect of glucocorticoids on osteoblast apoptosis. In contrast, no effect on osteoclast apoptosis is seen in the human skeleton (Dempster et al. 1997).

The lack of change in bone mineralization seen in the present study could be due to effects of glucocorticoids on osteoclasts. Dempster et al. (1997) have shown that glucocorticoids increase BMD in the rat and that the effect is attributed to increased osteoclast apoptosis which is in sharp contrast to the effect on the human skeleton.

Glucocorticoids are known to exert direct and complex effects on chondrocytes and osteoblasts and are therefore able to affect bone formation (Chen & Feldman 1979, Wong et al. 1990, Canalis 1996, Swolin et al. 1996, Klaus et al. 2000). Thus, the affected skeletal growth in male Dex-treated rats might involve direct effects on mesenchymal cells within the skeleton. Another possibility is that Dex may affect skeletal growth via a direct central effect on the brain and the HPA axis, as the time chosen for the Dex treatment corresponds to an early period when fetal brain development occurs in the rat (Nyrienda et al. 1998). It has previously been demonstrated that proper development of the fetal HPA axis is critical for normal growth and organ development. A third possibility is an indirect effect via the placenta or via hormones and growth factors in the pregnant dam. The observed effects may not be an effect of Dex but may be the result of changes in sensitivity to other hormones or the alteration in other factors as a result of Dex exposure.

Our study contributes to the investigation on the effects on skeletal growth and bone tissue in adult rat offspring. However, future studies are required to explore the complex effects and mechanisms of glucocorticoids on bone tissue after prenatal exposure, as the effect depends on the species, the duration of exposure, and the dose and time of exposure during pregnancy.

Leptin is involved in regulating food intake, energy balance, bone formation and neuroendocrine function. Increasing evidence indicates that leptin may play a role in fetal bone metabolism (Ogueh et al. 2000). Leptin has also been shown to have a potential role in endochondral ossification (Kume et al. 2002) and furthermore leptin reduces ovariectomy-induced bone loss in rats (Burguera et al. 2001). Recent data imply that leptin is involved in the central regulation of bone remodelling (Ducy et al. 2000) and the increased leptin levels in the present study may reflect a central mechanism for skeletal regulation. This idea is supported by a recent study demonstrating that
skeletal growth is increased in ob/ob mice (Steppan et al. 2000).

IGF-I is involved both in skeletal growth and in adult bone metabolism. IGF-I is regarded as an anabolic factor for bone formation (Canalis 1996). The proliferation of chondrocytes and the expression of IGF-I in the growth plate is affected by corticosteroids (Klaus et al. 2000). Furthermore, glucocorticoids have been shown to decrease the expression of IGF-I in human osteoblasts (Swolin et al. 1996). However, the affected skeletal growth in Dex-treated offspring was not associated with any effect on serum levels of IGF-I at the times examined. Whether the IGF-I levels were affected at the time of exposure remains unknown.

In conclusion, prenatal Dex exposure affects skeletal growth in rats in a gender-specific manner, while the mineralization of bones is unaffected in both male and female offspring.

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