Effects of In Utero and Lactational TCDD Exposure on Bone Development in Differentially Sensitive Rat Lines

Hanna M. Miettinen,*1 Pasi Pulkkinnen,† Timo Jämsä,† Jaana Koistinen,* Ulla Simanainen,* Jouko Tuomisto,*§ Juha Tuukkanen,‡ and Matti Viluksela*

*National Public Health Institute, Department of Environmental Health, FIN-70701 Kuopio, Finland; †Department of Medical Technology, University of Oulu, 90014 University of Oulu, Finland; ‡Department of Anatomy and Cell Biology, University of Oulu, FIN-90014 University of Oulu, Finland; §Department of Public Health and General Practice, University of Kuopio, FIN-70211 Kuopio, Finland

Received December 8, 2004; accepted February 19, 2005

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a notorious model compound of highly toxic environmental pollutants, polychlorinated dibenzo-p-dioxins (PCDDs). Their toxic effects are mediated via cytosolic aryl hydrocarbon receptor (AHR). We studied the effects of several dose levels of TCDD on developing rat bone after maternal exposure at different times of gestation and lactation in three differentially sensitive rat lines. Rat lines A, B, and C differ in their sensitivity to TCDD due to mutated AHR (Ahrbw) in line A and another TCDD-resistance allele (Bbw) in line B. Line C rats have no resistance alleles. Offspring were analyzed for bone mineral density and geometry by peripheral quantitative computed tomography (pQCT) and for bone biomechanics by three-point bending at mid-diaphysis of tibia and femur and by axial loading at femoral neck. TCDD treatment resulted in bone defects, mainly in offspring of the most sensitive line C at a maternal dose of 1 µg/kg. They included decreased bone length, cross-sectional area of cortex, and bone mineral density. Mechanical testing revealed significantly reduced bending breaking force and stiffness of tibia and femur and by axial loading at femoral neck. TCDD treatment resulted in bone defects, mainly in offspring of the most sensitive line C at a maternal dose of 1 µg/kg. They included decreased bone length, cross-sectional area of cortex, and bone mineral density. Mechanical testing revealed significantly reduced bending breaking force and stiffness of tibia and femur, and femoral neck. The effects were exposure time-dependent, and earlier exposure caused more severe defects. Gestational exposure alone was not sufficient, but lactational exposure was required to cause the bone defects. Most of the defects were recovered at the age of 1 year. The results indicate that dioxins affect developing bone by interfering with bone growth and mechanical strength and that the effects are mainly reversible. The dioxin-resistance alleles, Ahrbw and Bbw, increase the resistance to these defects.

Key Words: dioxin; development; bone; peripheral quantitative computed tomography; biomechanics.

INTRODUCTION

Polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs) are ubiquitous, highly toxic and stable environmental pollutants that enrich in food chains and may pose a threat to humans. They induce a variety of toxic effects in laboratory animals, ranging from wasting syndrome and metabolic disturbances to immunotoxicity, cancer, reproductive toxicity, and developmental defects (Pohjanvirta and Tuomisto, 1994). These effects are primarily mediated by the cytosolic aryl hydrocarbon receptor (AHR), which is a ligand-activated transcription factor with a basic helix-loop-helix motif (Schmidt and Bradfield, 1996). Although the sensitivity of adult animals to many dioxin effects varies greatly among animal species, sensitivity to developmental defects seems to occur at quite similar dose range in most species of laboratory animals studied so far (Birnbaum, 1995; Peterson et al., 1993). Developmental defects have been observed at very low exposure levels, indicating high dioxin sensitivity of developing organ systems in general. In addition, background exposure levels of breast-fed infants to dioxins has been estimated to be up to two orders of magnitude higher than in adults (Päpke, 1998). Therefore developmental defects are considered the most critical dioxin effects for human risk assessment (WHO, 2000), and impaired development of the reproductive system and teeth have been shown to belong to the most sensitive targets of perinatal PCDD/F exposure (Gray et al., 1997a; Kattainen et al., 2001; Mably et al., 1992a, 1992b). In these studies adipose tissue TCDD concentrations in dams at the lowest observable adverse effect levels (LOAELs) are estimated to be only slightly higher (about 177 pg TCDD/g fat based on (Hurst et al., 2000b)) than the higher end mother’s milk PCDD/F concentrations in the general population (about 100 pg I-TEQ/g fat, as reported by Alaluusua et al. [1996]). Mineralization defects of the molars have also been reported in children exposed to higher background PCDD/F levels via mother’s milk (Alaluusua et al., 1999).

We have recently reported that long-term exposure to TCDD dose-dependently interferes with bone growth, modeling, and mechanical strength in adult Long-Evans (Turku/L-E) and Han/Wistar (Kuopio; H/W) rats (Jämsä et al., 2001). Decreased tibial growth was associated with altered bone geometry, as

1 To whom correspondence should be addressed at National Public Health Institute, Department of Environmental Health, Laboratory of Toxicology, P.O. Box 95, FIN-70701 Kuopio, Finland. Fax: +358-17–201265. E-mail: hanna.miettinen@ktl.fi.
H/W rats have another, still unknown allele Bhw sensitivity. H/W rats have a point mutation in the Ahr residue 283 that results in loss of amino acids from the transactivation domain of the receptor protein and a high resistance to some but not all dioxin effects, and (4) to study the influence of the dioxin-resistance alleles. These lines exhibit highly different LD50 values for TCDD: >2000, 410, and 19 μg/kg in line A, B, and C females, respectively.

Because of the general sensitivity of developing animals to dioxins and the high potential dioxin exposure via mother’s milk, we studied the effects of low-dose prenatal and postnatal TCDD exposure on rat bone development. The studies were designed (1) to establish the dose–response relationships of possible bone effects, (2) to define the critical window of sensitivity during development, (3) to examine the reversibility of the effects, and (4) to study the influence of the dioxin-resistance alleles Ahhrhw and Bhw on sensitivity to these effects by using differentially sensitive line A, B, and C rats.

### MATERIALS AND METHODS

#### Chemicals

TCDD was >99% pure as determined by gas chromatography-mass spectrometry. It was dissolved in corn oil (Sigma Chemical). Dosing solutions were mixed in a magnetic stirrer and sonicated for 20 min before dosing.

#### Animals

Line A, B, and C rats were received from the SPF barrier unit of the National Public Health Institute (Kuopio, Finland). After mating, pregnant dams were kept in plastic Macrolon cages containing aspen-chip bedding (Tapvei Co, Kaavi, Finland) and covered with wire-mesh lids until the pups were weaned on postnatal day (PND) 28. The pups were kept in Macrolon cages similarly with their dams. The rats were kept under a photoperiodic cycle of 12 h light/12 h dark in an air-conditioned room. The mean temperature was 21±0.5°C and the relative humidity 40±7%. Pelleted rat feed (R36, Lactamin) and tap water were available ad libitum.

#### Experimental Design

Three different studies were carried out; a dose–response study in lines A, B, and C (Kattainen et al., 2001; Miettinen et al., 2002) was followed by a study with different timing of exposure in the most sensitive line C (Kattainen et al., 2001; Miettinen et al., 2002) and a one-year follow-up study in all three rat lines. Observations on tooth development in the first two studies have been previously published by Kattainen et al. (2001) and Miettinen et al. (2002), respectively, and observations on the male reproductive system development are presented in the first study by Simanainen et al. (2004).

Rats were mated overnight 1–2 females with one male, and the day sperm was deposited was assigned gestational day (GD) 0. Pregnant dams were given a single oral dose of 0.03, 0.1, 0.3, or 1 μg/kg TCDD in the dose–response study, and 1 μg/kg in the one-year follow-up study on GD15 at a volume of 4 ml/kg. The highest dose had to be limited to 1 μg/kg in GD15 at a volume of 4 ml/kg. The highest dose had to be limited to 1 μg/kg in TCDD-resistant lines A and B also, because a pilot study had revealed that a maternal dose of 3 μg/kg on GD15 resulted in 100% postnatal death of line A offspring. In the timing of exposure study, pregnant line C rats received a single dose of 1 μg/kg during gestation on GD11, GD13, or GD19, or after delivery on PND 0, PND2, or PND4 at volume of 4 ml/kg. Control rats received corn oil on GD15. For cross-fostering groups, two groups of dams from the timing of exposure study were dosed on GD15 with either TCDD or corn oil. Pups from dashed dams were transferred to control dams and vice versa on PND0, before the dams started to lactate. In all studies, the offspring number was recorded and litter size was adjusted to six animals (three males + three females) on PND1, if possible, and there were 3–6 litters in each group (2 in the TDCC group

<table>
<thead>
<tr>
<th>Dose μg/kg</th>
<th>Female offspring/litters</th>
<th>Exposure timing study</th>
<th>One-year follow-up study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Line A</td>
<td>Line B</td>
<td>Line C</td>
</tr>
<tr>
<td>0</td>
<td>7/6</td>
<td>6/4</td>
<td>8/3</td>
</tr>
<tr>
<td>0.1</td>
<td>6/4</td>
<td>7/5</td>
<td>8/3</td>
</tr>
<tr>
<td>0.3</td>
<td>6/4</td>
<td>6/5</td>
<td>7/5</td>
</tr>
<tr>
<td>1</td>
<td>7/6</td>
<td>6/5</td>
<td>8/3</td>
</tr>
<tr>
<td></td>
<td>PND0</td>
<td>8/5</td>
<td>8/5</td>
</tr>
<tr>
<td></td>
<td>PND2</td>
<td>8/5</td>
<td>8/5</td>
</tr>
<tr>
<td></td>
<td>PND4</td>
<td>8/5</td>
<td>8/5</td>
</tr>
<tr>
<td></td>
<td>IU</td>
<td>8/5</td>
<td>8/5</td>
</tr>
</tbody>
</table>

**Note.** IU group and L group in exposure timing study are cross-fostered groups. In IU group the exposure occurred between GD15 and birth via placenta, and in L group exposure between birth and weaning on PND28 via lactation.

Indicated by decreased cross-sectional and medullary areas at the diaphysis. Cortical bone mineral density (BMD) was not affected, but the three-point bending test indicated decreased bending breaking force and stiffness of the tibial diaphysis. These changes were observed at exposure levels that are not much higher than current average human dioxin exposures.

Interestingly, the dioxin-resistant H/W rats with mutated AHR (Pohjanvirta et al., 1998) were more resistant to these effects than L-E rats with normal AHR structure and high dioxin sensitivity. H/W rats have a point mutation in Ahr, which results in loss of amino acids from the transactivation domain of the receptor protein and a high resistance to some but not all end points of dioxin toxicity (Pohjanvirta et al., 1998; Simanainen et al., 2002; Tuomisto et al., 1999). In addition, H/W rats have another, still unknown allele Bhw that has a smaller influence on dioxin resistance. By selective crossing of H/W and L-E rat strains, three new rat lines were created, which differ in their sensitivity to short-term toxic effects of TCDD (Simanainen et al., 2003; Tuomisto et al., 1998, 1999). Line A rats have the altered H/W type AHR (Ahhrhw), line B rats have the other resistance allele Bhw, and line C rats have no resistance alleles. These lines exhibit highly different LD50 values for TCDD: >2000, 410, and 19 μg/kg in line A, B, and C females, respectively.

Because of the general sensitivity of developing animals to dioxins and the high potential dioxin exposure via mother’s milk, we studied the effects of low-dose prenatal and postnatal TCDD exposure on rat bone development. The studies were designed (1) to establish the dose–response relationships of possible bone effects, (2) to define the critical window of sensitivity during development, (3) to examine the reversibility of the effects, and (4) to study the influence of the dioxin resistance alleles Ahhrhw and Bhw on sensitivity to these effects by using differentially sensitive line A, B, and C rats.
exposed on PND13. In the exposure timing study, both male and female offspring were examined, but the other studies were carried out in female offspring. The offspring were weaned on PND28, after which they were housed with same-sex littermates. Body weight was measured weekly. The offspring were killed by CO2 asphyxiation and cervical dislocation on PND35 (dose–response study; only females studied), PND40 (exposure timing study; both males and females studied), or at the age of 52 weeks (one-year follow-up study; only females studied). Femurs and tibias were stored at −20°C until analysis. The bones were thawed at room temperature immediately before analysis, and the soft tissue was removed. Bone length was measured with a digital Vernier caliper.

**Bone densitometry.** The bones were scanned with a Stratec XCT 960 A pQCT system with software version 5.21 (Norland Stratec Medizintechnik GmbH). The bones were inserted into a plastic tube filled with 0.9% NaCl to position the samples for the measurements. A voxel size of 0.148 × 0.148 × 1.25 mm³ was used for the measurement. An attenuation threshold value of 0.7 cm⁻¹ was used to define cortical bone. The precision and accuracy of the pQCT system used had been verified previously (Jäämsi et al., 2001). One cross-sectional slice from each bone was scanned at midshaft, which was determined from the scout view of the pQCT system. Cross-sectional area of cortex (CSA) and cortical bone mineral density (BMD), polar cross-sectional moment of inertia (PMI), and periosteal and endosteal circumference (PERI and ENDO, respectively) were measured at the midshaft of the bone.

**Mechanical testing.** After the pQCT measurements, the bones were subjected to mechanical testing, as described elsewhere (Jäämsi et al., 1998; Peng et al., 1994). Briefly, the materials testing machine (Jäämsi et al., 1998) with amplifier (Jäämsi and Jalovaara, 1996) and force sensor (Gefran TU KSD, 0–50 kg, Gefran Sensori) was used to measure the failure load of the three-point bending strength of tibia, femur, and femoral neck. In the three-point bending test, the bone was placed on a supporter with two loading points, 13 mm apart. The pressing force was directed vertically to the midshaft of the bone. To measure the failure load of the femoral neck, the proximal half of the femur was placed axially into a suitable hole on the supporter and pressed in a direction parallel to the femoral shaft. The constant compression speed of 0.155 mm/s was used in both configurations. A laboratory plotter (Yokogawa LR 102, Yokogawa Europe) recorded the compression load in dependence on the time. The load–deformation relationship was obtained by conversion of the load–time curve. The maximal load (N) was used for evaluation of bone strength and the stiffness (N/mm) was calculated according to the slope of the linear part of the curve.

**Analysis of TCDD tissue concentrations.** Pregnant line C females were exposed to 0.5 μg/kg TCDD on GOD, GD11 or GD15 (3–5 rats per group), at volume of 4 ml/kg. On GD22, females were monitored every half-hour and born offspring were moved from the cage to prevent suckling. Dams were killed with CO2 asphyxiation and cervical dislocation and offspring with an overdose of pentobarbital (Mebunal, Orion Pharma). One group of rats exposed on GD15 was allowed to rear offspring until PND5, when the animals were killed and studied. Offspring were frozen as a whole at −20°C until homogenization with Bamix M133 mixer (ESGE AG). Each litter was homogenized to gain a litter sample. The same amount of homogenized offspring tissue from each litter was then pooled to gain a group sample. The same amount of perirenal adipose tissue from each dam was pooled for a group fat sample. Tissue samples were freeze-dried and extracted in a Soxhlet apparatus with toluene for 18 h. The solvent was changed to hexane and the fat% was determined gravimetrically.

The extract or an aliquot was spiked with an internal standard solution containing ¹³C-labeled TCDD and was purified using silica gel, carbon, and aluminum oxide columns (Vartiainen et al., 1995). Prior to analyses of TCDD by gas chromatography/mass spectrometry (GC/MS), the purified extract was spiked with a recovery standard solution containing ¹³C-1,2,3,4-TCDD and was concentrated with nitrogen flow to final volume of 30 μl nanone.

GC/MS analyses were carried out with a VG 70–250SE high-resolution mass spectrometer (VG Analytical) interfaced to a HP 6890 high-resolution gas chromatograph (Hewlett-Packard). The mass spectrometer was operated in the selected ion monitoring (SIM) mode at a resolution of 10,000 in electron impact ionization (EI) mode (35 eV). Two ions of the molecular ion cluster (M⁺ and (M+2)⁺) were recorded for each followed compound.

Identification of 2,3,7,8-TCDD was verified by a comparison of the GC retention time and ion ratios with those of the reference compound. Detection limits were as follows: liver 0.1 pg/g fresh weight, lipid tissue 0.5–5 pg/g lipid weight.

**Statistics.** A femur and a tibia of one female and one male per litter were examined; otherwise, additional samples were studied from randomly selected litters to gain a minimum of six studied offspring. Because of the small number of offspring in groups exposed on GD11 and GD13, all of them were studied. The results were analyzed on a litter basis, maintaining litter independence. The parameters were tested with the analysis of variance (ANOVA) followed by the least significant difference (LSD) test in the cases where the data displayed normal distribution (Levene’s test); otherwise, the nonparametric Kruskal-Wallis ANOVA was used, followed by the Mann-Whitney U-test. The limit of statistical significance was set at p < 0.05.

**RESULTS**

**Body Weight Development and Survival**

No maternal toxicity was observed, and the maternal body weight gain during pregnancy was not affected by TCDD treatment in any rat line. In offspring, TCDD exposure at 1 μg/kg decreased body weights in line B on PNDs 1–7 and in line C throughout the dose–response study (Kattainen et al., 2001; Miettinen et al., 2002). In the exposure timing study, the reduction was the greater the earlier the dam was exposed (Kattainen et al., 2001; Miettinen et al., 2002). In the one-year follow-up study, exposed line B female offspring weighed slightly (about 8%) but significantly less than controls on weeks 7–11 (data not shown). Exposure to 1 μg/kg TCDD on GD11 and GD13 resulted in increased mortality in line C offspring (Miettinen et al., 2002). In the other lines, no signs of toxicity were detected.

**Bone Length and Geometry**

Changes in bone length and geometry were observed only in line C rats and only at the highest maternal dose of 1 μg/kg TCDD (Table 2; data for lines A and B not shown). The length of tibia and femurs of line C offspring exposed to 1 μg/kg TCDD was slightly but nonsignificantly shorter than that of control offspring in the dose–response study. In the exposure timing study, tibia was significantly shorter in both genders exposed on GD11 and GD13, as well as in females exposed on GD19 and in males in the cross-fostered group exposed only via lactation. Femur length was significantly shorter only in males exposed on GD11, GD19, PND2 and in both cross-fostered groups as compared to controls. Cross-sectional area of femoral cortex (CSA) was decreased in line C at 1 μg/kg TCDD in the dose–response study (Table 2, Fig. 1). In the exposure timing study, tibial and femoral CSA was decreased in males and tibial CSA was decreased in females exposed on GD11, GD 13, and GD19. Dose-dependent decrease in
## Table 2

Effects of TCDD on Bone Geometry and Densitometry in Line C Rats

<table>
<thead>
<tr>
<th></th>
<th>Tibia</th>
<th>Femur</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (mm)</td>
<td>CSA (mm²)</td>
</tr>
<tr>
<td><strong>Dose-response study Maternal dose (µg/kg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>25.3 ± 0.6</td>
<td>1.57 ± 0.09</td>
</tr>
<tr>
<td>0.1</td>
<td>26.3 ± 0.1</td>
<td>1.54 ± 0.09</td>
</tr>
<tr>
<td>0.3</td>
<td>25.0 ± 0.4</td>
<td>1.65 ± 0.09</td>
</tr>
<tr>
<td>1</td>
<td>22.8 ± 1.6</td>
<td>1.22 ± 0.18</td>
</tr>
</tbody>
</table>

**Exposure timing study Males**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GD11</th>
<th>GD13</th>
<th>GD19</th>
<th>PND0</th>
<th>PND2</th>
<th>PND4</th>
<th>IU</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0</strong></td>
<td>28.5 ± 0.3</td>
<td>23.0 ± 0.3*</td>
<td>23.2 ± 1.8*</td>
<td>25.5 ± 0.7</td>
<td>26.8 ± 1.4</td>
<td>26.7 ± 0.6</td>
<td>26.7 ± 0.6</td>
<td><strong>25.6 ± 1.0</strong></td>
<td>2.40 ± 0.16</td>
</tr>
<tr>
<td><strong>0.1</strong></td>
<td>27.7 ± 0.1</td>
<td>25.1 ± 0.1*</td>
<td>26.0 ± 0.4*</td>
<td>27.5 ± 0.7</td>
<td>28.7 ± 0.4</td>
<td>28.4 ± 0.6</td>
<td>28.4 ± 0.6</td>
<td>27.8 ± 0.8</td>
<td>2.39 ± 0.16</td>
</tr>
<tr>
<td><strong>0.3</strong></td>
<td>27.0 ± 0.4</td>
<td>26.2 ± 0.3*</td>
<td>26.8 ± 0.3*</td>
<td>27.9 ± 0.7</td>
<td>28.3 ± 0.4</td>
<td>28.1 ± 0.6</td>
<td>28.1 ± 0.6</td>
<td>27.5 ± 0.8</td>
<td>2.34 ± 0.16</td>
</tr>
<tr>
<td><strong>1</strong></td>
<td>26.9 ± 0.4</td>
<td>25.9 ± 0.1*</td>
<td>26.6 ± 0.3*</td>
<td>28.0 ± 0.7</td>
<td>28.4 ± 0.4</td>
<td>28.2 ± 0.6</td>
<td>28.2 ± 0.6</td>
<td>27.6 ± 0.8</td>
<td>2.32 ± 0.16</td>
</tr>
</tbody>
</table>

**Exposure timing study Females**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GD11</th>
<th>GD13</th>
<th>GD19</th>
<th>PND0</th>
<th>PND2</th>
<th>PND4</th>
<th>IU</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0</strong></td>
<td>27.3 ± 0.4</td>
<td>22.5 ± 0.4*</td>
<td>22.5 ± 1.3*</td>
<td>24.2 ± 1.1*</td>
<td>24.8 ± 2.1</td>
<td>25.0 ± 0.9</td>
<td>26.4 ± 0.5</td>
<td><strong>25.6 ± 1.0</strong></td>
<td>2.40 ± 0.16</td>
</tr>
<tr>
<td><strong>0.1</strong></td>
<td>26.5 ± 0.4</td>
<td>21.9 ± 0.1*</td>
<td>22.3 ± 0.3*</td>
<td>23.9 ± 1.5</td>
<td>24.3 ± 2.0</td>
<td>25.0 ± 0.9</td>
<td>26.4 ± 0.5</td>
<td>25.6 ± 1.0</td>
<td>2.40 ± 0.16</td>
</tr>
<tr>
<td><strong>0.3</strong></td>
<td>25.9 ± 0.4</td>
<td>21.4 ± 0.1*</td>
<td>22.1 ± 0.3*</td>
<td>23.6 ± 1.5</td>
<td>24.1 ± 2.0</td>
<td>24.8 ± 0.9</td>
<td>26.2 ± 0.5</td>
<td>25.6 ± 1.0</td>
<td>2.40 ± 0.16</td>
</tr>
<tr>
<td><strong>1</strong></td>
<td>25.4 ± 0.4</td>
<td>20.9 ± 0.1*</td>
<td>21.5 ± 0.3*</td>
<td>23.0 ± 1.5</td>
<td>23.5 ± 2.0</td>
<td>24.2 ± 0.9</td>
<td>25.6 ± 0.5</td>
<td>25.6 ± 1.0</td>
<td>2.40 ± 0.16</td>
</tr>
</tbody>
</table>

**One-year follow-up study**

|                | 0       | 36.1 ± 0.5 | 4.88 ± 0.24 | 8.98 ± 0.19 | 4.40 ± 0.08 | 1239 ± 21 | 5.68 ± 0.45 | 31.0 ± 0.53 | 7.49 ± 0.45 | 11.88 ± 0.23 | 6.85 ± 0.29 | 1294 ± 22 | 16.52 ± 1.59 |
|----------------|---------|------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| 1              | 36.8 ± 1.2 | 4.64 ± 0.02 | 8.55 ± 0.04 | 3.85 ± 0.07 | 1225 ± 16 | 4.65 ± 0.07 | 30.6 ± 0.25 | 7.43 ± 0.03 | 11.67 ± 0.11 | 6.54 ± 0.16 | 1283 ± 19 | 15.71 ± 0.75 |

**Note.** Values are given as mean ± S.E. *p < 0.05 as compared to controls (ANOVA + LSD). CSA: cross-sectional area of cortex; PERI: periosteal circumference; ENDO: endosteal circumference; BMD: cortical bone mineral density; PMI: polar cross-sectional moment of inertia.
medullary area, as indicated by smaller endosteal circumference (ENDO), was seen in both long bones, and the decrease was significant at 1 \mu g/kg in femur. In the exposure timing study, ENDO and periosteal circumference (PERI) in femur were decreased in both genders, though not significantly in all groups. In tibia, PERI was significantly decreased only in females in all groups except PND4. Polar cross-sectional moment of inertia (PMI) was decreased in femurs of line C rats at 1 \mu g/kg in both genders, although statistical significance was not found in all groups, and results were similar in tibia of females in the time-exposure study.

**Bone Mineral Density**

In the dose–response study, cortical bone mineral density (BMD) of tibia and femur of line C females was significantly reduced at 1 \mu g/kg TCDD (Table 2). In line A and B rats, no significant changes were found among reported parameters (data not shown). In the exposure timing study, BMD of both long bones of female offspring was reduced in the group exposed on GD11 and on GD19. In males, femoral BMD was significantly smaller in all groups where exposure started in utero and continued via lactation and tibial BMD was significantly smaller in groups exposed on GD11 and GD13.

**Mechanical Parameters**

TCDD-treatment resulted in consistent changes in the bone mechanical parameters only in line C rats (Table 3, Fig. 3; data for line A and B rats not shown).

**Females**

In the dose–response study, breaking force of tibia and femoral neck as well as tibial stiffness were significantly reduced at 1 \mu g/kg (Table 3). Breaking force of long bones was significantly reduced only in groups exposed both in utero and via lactation (Fig. 3).

**Males**

In male offspring, the breaking force of tibia and femur was significantly reduced in groups exposed on GD11 and GD13. The breaking force of femoral neck was also slightly but nonsignificantly decreased. The only significant change in bending stiffness was found in femur in groups GD11, GD13, and PND2.

**Gender Differences**

No significant differences between males and females were observed in bone geometry, densitometry, or mechanical parameters in the exposure timing study groups where both genders were examined.

**TABLE 3**

<table>
<thead>
<tr>
<th>Maternal dose (\mu g/kg)</th>
<th>Tibia</th>
<th>Femur</th>
<th>Femoral neck</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Breaking force (N)</td>
<td>Stiffness (N/mm)</td>
<td>Breaking force (N)</td>
</tr>
<tr>
<td>Dose–response study 0</td>
<td>29.4 ± 2.4</td>
<td>69.5 ± 6.2</td>
<td>29.8 ± 4.2</td>
</tr>
<tr>
<td>0.1</td>
<td>29.7 ± 1.7</td>
<td>78.4 ± 6.5</td>
<td>29.5 ± 0.8</td>
</tr>
<tr>
<td>0.3</td>
<td>28.0 ± 1.7</td>
<td>77.6 ± 4.1</td>
<td>31.2 ± 1.3</td>
</tr>
<tr>
<td>1.0</td>
<td><strong>18.7 ± 3.5</strong></td>
<td><strong>48.8 ± 9.9</strong></td>
<td><strong>24.6 ± 4.4</strong></td>
</tr>
<tr>
<td>One-year follow-up study 0</td>
<td>123.5 ± 7.6</td>
<td>283.2 ± 28.7</td>
<td>195.0 ± 25.0</td>
</tr>
<tr>
<td>1</td>
<td>117.7 ± 4.1</td>
<td>249.6 ± 11.3</td>
<td>171.3 ± 6.6</td>
</tr>
</tbody>
</table>

*Note. Values are given as mean ± S.E. *p < 0.05 as compared to controls (ANOVA + LSD).*
Reversibility of the Effects

The one-year follow-up study showed that most of the bone effects induced by in utero and lactational exposure to TCDD in line C rats were reversed at the age of 1 year. Tibial and femoral length as well as BMD had returned to normal during the 1-year observation period. Of the geometric changes, tibial ENDO and PERI circumferences as well as PMI were still decreased, but without statistical significance (Table 2). Similarly, mechanical strength of exposed 1-year-old line C rat bones was slightly but nonsignificantly reduced compared to controls (Table 3).

TCDD Concentration in Newborn and Dam

Fresh weight-based and lipid-adjusted average TCDD tissue concentrations of offspring and maternal adipose tissue TCDD concentrations analyzed on PND0 are shown in Figure 2. The average body weight of newborns on PND0 was 5.2, 5.3, 5.7,
and 5.5 g in GD8, GD11, GD15, and control groups, respectively, and the average fat percentages were 1.1%, 1.3%, 1.4%, and 1.1%, respectively. Body weight and fat percentage of offspring studied on PND5 were 9.3 g and 7.7%, respectively. TCDD concentrations were the lower the earlier the dams were exposed, reflecting elimination. Lactation resulted in considerably increased accumulation of TCDD as the average tissue concentration of the offspring (on fresh weight basis) was an order of magnitude higher on PND5 than on PND0 (Fig. 2).

**DISCUSSION**

We studied the effects of TCDD on rat bone development using pQCT and mechanical analyses. TCDD-treatment resulted in adverse changes in three aspects of bone quality: bone geometry, bone mineral density, and bone mechanical properties. These changes were dependent on the timing of exposure and the dose of TCDD, and there was only a partial recovery. In addition, the dioxin resistance alleles Ahfr" and Bb" increased the resistance of rats to these effects, as virtually no adverse effects were observed in the bones of the control groups at the used dose levels.

Exposure times were selected so that all phases of bone development were covered, as the earliest time of exposure (GD11) was 2 days before the first signs of skeletal system development are detectable and the last time of exposure was 4 days after birth. A single maternal dose of TCDD is sufficient to cover the whole prenatal and neonatal period, as the elimination half-life of TCDD is about 26 days in female rats (Li et al., 1995) The development of rat skeleton begins on GD13 as a condensation of mesenchymal tissue (Hebel and Stromberg, 1986). Two days later, cartilaginous structures appear in skeleton and by GD17 ossification of the limb bones is observable. The ossification proceeds rapidly, and 2 days after birth all the skeletal elements are ossified (Fritz and Hess, 1970). Ossification continues after birth as bones grow, and in rats epiphyseal cartilages remain open until senescence. Our results showed that the early TCDD exposure on GD11 resulted in the most severe bone effects. Therefore, the sensitive period for bone effects starts from the initial phase of bone development and the sensitivity decreases during later stages of development. Although the contribution of gestational exposure via placenta to the TCDD body burden of a weanling rat is negligible compared to lactational exposure (Hurst et al., 2000a; Li et al., 1995), combined exposure during pregnancy and via lactation was clearly more effective than only lactational exposure starting after birth. This may be so because ossification of rat skeleton is already nearly complete at term (Fritz and Hess, 1970). Bone growth is based on further ossification after birth, but it seems to be more resistant to TCDD than the earlier phases of bone formation (cf. Jämäsa et al., 2001). The most sensitive period of bone development has therefore passed before the higher exposure via lactation starts. However, gestational exposure alone did not cause severe bone defects. In the cross-fostered groups, the effects were limited to bone geometry, as no remarkable alterations were found in mechanical properties of the bones. It is interesting to note that, in spite of different timing, the lower TCDD exposure during gestation resulted in very similar bone effects to higher lactational exposure during the neonatal period. Therefore both gestational and postnatal exposures are required for the complete spectrum of TCDD-induced adverse effects in bone development.

We have previously shown that in adult rats bone effects are observed after repeated and prolonged TCDD treatment and they require higher dose than developing rats (Jämäsa et al., 2001). Treatment of 10-week-old rats with weekly doses of TCDD for 20 weeks resulted in quite similar changes with those found in the present study, i.e., altered bone geometry and decreased mechanical strength, but no BMD changes. In TCDD-sensitive adult L-E rats, bone effects were observed at the total dose level of 1.7 μg/kg TCDD and above, whereas in this study the effects were limited to the offspring of TCDD-sensitive line C rats after a single maternal dose of 1 μg/kg. Analysis of TCDD tissue concentration indicated that TCDD body burden (average tissue concentration) of the offspring was only about 5% of the initial maternal dosage (0.5 μg/kg) on PND0 and about 54% on PND5. However, TCDD concentrations in lipid - that are in equilibrium with toxicologically effective concentrations - were quite similar in dams and offspring during gestation. Despite fundamentally different dosing schemes in adult study and in the present study.

Although rat bones proved to be more sensitive to TCDD during perinatal development than in adulthood, the changes observed were qualitatively rather similar in spite of different stages of development at exposure. The only remarkable difference between in utero/lactational and adult (Jämäsa et al., 2001) TCDD exposure was in BMD. Interestingly, decreased volumetric BMD was observed only in rats whose TCDD exposure started prenatally, and the decrease in BMD was the greater the earlier the rat was exposed. In addition, the decreased BMD returned to normal levels during the first year of life. In previous studies, exposure of adult rats to dioxin-like coplanar polychlorinated biphenyl (PCB)126 did not affect BMD at all (Lind et al., 1999, 2000b) or resulted in increased BMD (Lind et al., 2000a).

Decreased length of long bones had been observed in rats exposed to TCDD (Jämäsa et al., 2001) or PCB126 (Lind et al., 1999, 2000b) during adulthood. The present data indicate that impaired bone growth is reversible after elimination of TCDD from the body. Of the bone geometric parameters CSA, ENDO, and PMI were decreased in juvenile line C rats at 1 μg/kg. We observed similar decreases in CSA and ENDO also in the tibial diaphysis of L-E rats exposed to TCDD in adulthood (Jämäsa et al., 2001).

Mechanical testing of juvenile line C rats revealed significant decreases in mechanical strength of tibial and femoral...
diaphysis as well as femoral neck, and this effect was most consistent in rats with prenatal exposure. This is also in line with our previous report from adult rats, whose tibial breaking force and bending stiffness were decreased in response to TCDD (Jämsä et al., 2001; Lind et al., 2000b). The one-year follow-up study revealed that there was still a slight, although statistically nonsignificant decrease in mechanical properties 1 year after birth. Diaphyseal bending strength and stiffness are absolute measures of bone mechanical strength, and they are strongly associated with the combination of geometrical and qualitative properties of bone (Jämsä et al., 1998) that were also diminished in line with bone mineral density. Therefore, bone mechanical strength reflects the TCDD-induced changes observed in bone geometry and BMD.

Studies in mice lacking the functional AHR have confirmed its essential role in mediating toxic effects of dioxins (Bunger et al., 2003; Fernandez-Salgueiro et al., 1996; Mimura et al., 1997; Peters et al., 1999). The characteristic TCDD-induced developmental defects cleft palate and hydromphrosis are not induced in these mice. AHR seems also to play a physiological role in bone development, because at fetal examination AHR induced in these mice. AHR seems also to play a physiological role in bone development, because at fetal examination AHR knockout mice had a lower incidence of large interfrontal developmental defects cleft palate and hydronephrosis are not observed in bone geometry and BMD. Therefore, bone mechanical strength reflects the TCDD-induced changes observed in bone geometry and BMD.

The presence of the AHR signaling pathway in developing bones suggests that the observed bone effects of TCDD may be directly mediated via AHR. Inhibited differentiation of cultured fetal rat calvarial osteoblasts exposed to 10 nM TCDD in vitro (Gierthy et al., 1994) speak for a direct effect on bone cells. In the present study the maternal dose of 0.5 μg/kg resulted in fresh weight based TCDD concentrations of 27 and 276 pg/g (=0.86 nM) in groups exposed on GD15 and analyzed on PND0 and PND15, respectively. As the bone effects were observed at the maternal dose of 1 μg/kg, the corresponding tissue concentrations are about 0.17 and 1.71 nM. Therefore the tissue concentration in our study (on GD5) was 5.8-fold lower than the concentration used by Gierthy et al. (1994), and as the TCDD body burden is likely to continue to increase during lactation, it will become more similar toward the end of the lactation period. This shows that the TCDD concentrations causing bone effects in vivo and in vitro are of similar magnitude.

A possible mechanism or contributing factor for dioxin-induced developmental bone effects is modulated cross talk between AHR and estrogen receptor (ER) α and β signaling pathways. Estrogen signaling is important for normal bone development and homeostasis, and TCDD has a variety of antiestrogenic effects from a complex modulation of cross talk between AHR and ERs (Kiets et al., 2004; Nilsson et al., 2001; Ohtake et al., 2003). It remains to be clarified whether this type of modulation also takes place under the conditions of the present study, i.e., in fetal and neonatal rats in vivo at very low dose in utero and lactational TCDD exposure.

In addition to direct effects on bone cells mediated by AHR or AHR/ER modulation, dioxin-induced alterations in hormonal and nutritional status may be a secondary mechanism of developmental bone toxicity. Estradiol levels were unaffected by TCDD in both developing and adult rats (Gray et al., 1997b; Pohjanvirta and Tuomisto, 1994). In agreement with these findings, our present and earlier results (Jämsä et al., 2001) indicate that the effects of TCDD on bone are fundamentally different from those of estrogen deficiency. Moreover, dioxin-like PCB126 with antiestrogenic effects did not cause trabecular bone loss and the increasing bone dimensions that are typical effects of estrogen deficiency (Lind et al., 1999). Although exposure of adult animals to higher doses of TCDD results in decreased circulating testosterone and dihydrotestosterone levels, no changes in hormone levels were found in male Holzman rats exposed to a single dose of up to 0.8 μg/kg TCDD on GD15 (Ohsako et al., 2001). These findings, and the fact that bone effects were observed at low dose levels that are not generally associated with hormonal alterations, suggest that they are not the primary cause of dioxin-induced bone toxicity. However, hormonal and nutritional factors potentially affecting bone modeling and remodeling have not yet been systematically monitored in developing TCDD-treated animals.

Previous studies in dioxin-resistant H/W, line A, and line B rats have shown that the resistance to several end points of dioxin toxicity is linked to the altered AHR C-terminal transactivation domain (Jämsä et al., 2001; Kattainen et al., 2001; Pohjanvirta et al., 1997; Simanainen et al., 2002, 2003; Tuomisto et al., 1998; Viluksela et al., 2000). Interestingly, dioxin resistance associated with resistance alleles Ahb′w and B′w is end-point–dependent and, based on short-term dioxin toxicity, they can be classified into two categories (Simanainen et al., 2002, 2003; Tuomisto et al., 1999). Type I end points (e.g., increased CYP1A activity, thymic involution) show similar sensitivity in dioxin-sensitive (“normal”) rats and in dioxin-resistant (H/W and line A) rats, and are independent of genotype variation. On the other hand, for type II end points (e.g., weight loss, liver toxicity, increased serum bilirubin), the efficacy (magnitude of effect) of TCDD is suppressed by the resistance alleles. The lack of effects in line A and B rats in the present study classifies the developmental bone toxicity to the type II category. This is in accordance with the previous findings on TCDD bone effects in adult H/W and L-E rats (Jämsä et al., 2001). Of other developmental end points of dioxin toxicity prevention of the third molar development (Kattainen et al., 2001), and reduced sperm counts (Simanainen et al., 2004) are also influenced by the resistance alleles.

In conclusion, the present study demonstrated that low perinatal TCDD exposure results in adverse changes in three aspects of bone quality: bone geometry, bone mineral density, and bone mechanical properties. Bone toxicity belongs to the
group of sensitive developmental end points of dioxin toxicity, together with impaired development of the reproductive system and teeth. TCDD exposure covering the early phases of bone development results in more severe effects than later exposure. There is a marked, but not complete recovery during 1 year. In addition, the dioxin-resistance alleles Ahrβsfn and Bβsfn increase the resistance of rats to the bone effects, which indicate that they belong to dioxin type II effects. However, almost complete lack of bone effects in line A and B rats did not allow quantification of the influence of resistance alleles. Future studies should examine dioxin effects on bone cells and on their differentiation, as well as the role of hormonal and nutritional status in these effects.

ACKNOWLEDGMENTS

We thank Jaana Jääskö, Arja Tamminen, and Virpi Tiihonen for excellent technical assistance. This study was financially supported by the Academy of Finland, Research Program for Environmental Health (Contract No 42551) and the European Commission, Contracts QLK4-CT-2002–02528 (BONE-TOX) and QLK4-CT-1999–01446.

REFERENCES


Lind, P. M., Larsson, S., Johansson, S., Mellhus, H., Wikstrom, M., Lindhe, O., and Orberg, J. (2000a). Bone tissue composition, dimensions and strength in female rats given an increased dietary level of vitamin A or exposed to 3',3', 4',5'-pentachlorobiphenyl (PCB126) alone or in combination with vitamin C. Toxicology 151, 11–23.


