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Effects of developmental exposure to perfluorooctanoic acid (PFOA) on long bone morphology and bone cell differentiation

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ABSTRACT

Perfluorooctanoic acid (PFOA) is a ubiquitous and persistent environmental chemical, which has been used extensively due to its stability and surface tension-lowering properties. Toxicological effects include induction of neonatal mortality and reproductive toxicity. In this study, pregnant C57BL/6 mice were exposed orally to 0.3 mg PFOA/kg/day throughout pregnancy, and female offspring were studied at the age of 13 or 17 months. Morphometrical and biomechanical properties of femurs and tibias were analyzed with micro-computed tomography and 3-point bending, and bone PFOA concentrations were determined by mass spectrometry. The effects of PFOA on bone cell differentiation were studied in osteoclasts from C57BL/6 mice and in the MC3T3 pre-osteoblast cell line. PFOA exposed mice showed increased femoral periosteal area as well as decreased mineral density of tibias. Biomechanical properties of these bones were not affected. Bone PFOA concentrations were clearly elevated even at the age of 17 months. In osteoblasts, low concentrations of PFOA increased osteocalcin (OCN) expression and calcium secretion, but at PFOA concentrations of 100 μM and above osteocalcin (OCN) expression and calcium secretion were decreased. The number of osteoclasts was increased at all PFOA concentrations tested and resorption activity dose-dependently increased from 0.1–1.0 μM, but decreased at higher concentrations. The results show that PFOA accumulates in bone and is present in bones until the old age. PFOA has the potential to influence bone turnover over a long period of time. Therefore bone is a target tissue for PFOA, and altered bone geometry and mineral density seem to persist throughout the life of the animal.

1. Introduction

Perfluoroalkyl substances (PFAS) are highly fluorinated aliphatic substances, the most common of which are the compounds perfluorooctanoic acid (PFOA) and perfluorooctanoic sulfonic acid (PFOS) (EFSA, 2008, 2012). They have been widely used in surfactant and polymer industries, for example in textile stain repellents and fire-extinguishing foams, because of their stability and surface tension-lowering properties due to their amphiphilic nature (Buck et al., 2011). Numerous studies have found these compounds widely distributed in sediments, drinking water supplies as well as in wildlife and in human tissues (Ehresman et al., 2007; Sakurai et al., 2010; Taniyasu et al., 2003). Extensive use of PFOA and PFOS in fire fighting foams has resulted in contamination of ground water supplies, for example in Sweden and in Germany (Weiss et al., 2012). Their toxicological properties include liver enlargement, spleen atrophy, reproduction and neonatal mortality (Post et al., 2012), which have led to the addition of PFOS and PFOA to the Annex B of the Stockholm Convention on Persistent Organic Pollutants. Recently, the European Chemical Agency (ECHA) proposed classification of PFOA as a substance of very high concern (SVHC) (ECHA, 2013).

PFOA is rapidly absorbed from the gastrointestinal tract and lungs, but also to some extent through the skin (reviewed by (Kennedy et al., 2004; Kudo and Kawashima, 2003; Lau et al., 2007). It is predominantly distributed to blood, liver and kidney, but also to bones and bone marrow (see below). PFOA crosses readily the placenta and is excreted into the milk (Hinderliter et al., 2005). PFOA is not metabolized in mammals, but it has been shown to undergo an extensive enterohepatic circulation. The elimination half-life of PFOA is about 4 years in humans and about 18 days in mice.

PFOA is an agonist of the peroxisome proliferator-activated receptor α (PPAR-α), and to some extent PPAR-γ and β/δ (Maloney and Waxman, 1999; Takacs and Abbott, 2007; Vanden Heuvel et al., 2006).

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These nuclear receptors have been linked to lipid metabolism, energy homeostasis and cell differentiation among others (Poulsen et al., 2012). Studies using knockout mice have indicated that the toxic effects of PFOA are mostly mediated by the PPAR-α receptor subtype (Wolf et al., 2008; Yang et al., 2002). Bone is a potential target for PPAR agonists as PPARs are expressed in osteoblasts and osteoclasts (Chan et al., 2007; Giaginis et al., 2007). Still et al. (2008) observed that linoleic acid and bezafibrate, the known agonists of PPAR-α/δ, increased significantly the number of osteoblasts and bone turnover in distal tibial metaphyses while the number of osteoclasts remained unaltered. On the other hand, some PPAR agonists have been shown to interfere with bone resorption as bezafibrate inhibited bone resorption by 50%, and other agonists, such as GW9578, L165041 and ciglitazone, inhibited the formation of osteoclasts (Chan et al., 2007). The fact that in male PPAR-α knockout mice the medullary area of femur was increased (Wu et al., 2000) indicates the role of PPARs in bone remodeling.

Bone is a highly mineralized tissue in which the action of osteoblasts forming bone in several steps during their maturation is coupled with the bone resorbing activity of osteoclasts. Disturbances in this balance can lead to various bone diseases, such as osteoporosis and osteopetrosis. Many environmental chemicals, for example dioxins and organotins, have been found to affect bone tissue *in vivo* and bone cells *in vitro* (Finnilä et al., 2010; Jämsä et al., 2001; Korkalainen et al., 2009; Koskela et al., 2012; Miettinen et al., 2005; Tsukamoto et al., 2004). In addition, PFOS and a shorter carbon chain perfluorinated compound PBSF (perfluorobutane sulfonate) have been shown to accumulate in bone and bone marrow (Bogdanska et al., 2011, 2014; Borg et al., 2010). Recently PFASs including PFOA were found in 20 human rib bone samples that had been collected at autopsy from individuals with a mean age of 56 (Perez et al., 2013). Reduced ossification was reported in mouse fetuses exposed during pregnancy to high dose levels of PFOA causing fetal toxic effects ranging from developmental delays to full-litter resorptions and neonatal mortality (Lau et al., 2006). In two recent studies a negative association between serum PFOS concentration and bone mineral density was observed in samples representing the adult US population (Lin et al., 2014; Khalil et al., 2015), although for PFOA the association was significant only for women of the latter study. These findings and the ability of other PPAR agonists to interfere with bone homeostasis led us to investigate whether bone acts as a target tissue for PFOA and whether exposure during pregnancy and infancy can cause long-term effects on bone.

In this study we analyzed the effects of *in utero* and lactational exposure to PFOA on mouse bones using micro-computed tomography (μCT) analysis to study the morphometry of bones, 3-point bending to evaluate bone strength and mass spectrometry to assess the concentration of PFOA in bones. In addition, using bone marrow mesenchymal stem cells from mice, we further characterized the effects of PFOA on osteoclast and osteoblast differentiation and function.

2. Materials and methods

2.1. Animal experiments and dissection

Samples of this study originated from an earlier experiment carried out for studying the effects of *in utero*/lactational exposure to PFOS and PFOA on motor function of mice (Onishchenko et al., 2011). All experiments were performed in accordance with the rules of the Swedish animal protection legislation and were approved by the local Animal Ethics Committee (Stockholms Norra Djurförsöksetiska Nämnd). Briefly, C57BL/6/Bkl female mice (Scanbur BK, Sweden) were mated with males overnight and the next morning was considered gestation day (GD) 1 if a vaginal plug was observed. Pregnant dams received PFOA ($n = 6$) (purity 96%, Sigma-Aldrich) mixed with food at the dose of 0.3 mg/kg/day throughout the pregnancy starting from GD 1 (total dose $0.3 \times 21 = 6.3$ mg/kg). The daily dose was selected so that it would not affect survival or cause general toxicity. It has been shown earlier that the benchmark dose lower confidence limit ($BMDL_5$) of PFOA in mice is 1.09 mg/kg/day for decreased neonatal survival and 0.86 mg/kg/day) for decreased postnatal body weight gain (Lau et al., 2006). PFOA, dissolved in 95% ethanol at a concentration of 1 μg/μl, was applied on palatable food in a volume adjusted according to the individual body weight. Control mice received pellets treated with 95% ethanol alone. The food pellets were kept on a bench for 2 h before they were placed in the cages to allow the ethanol to evaporate. On postnatal day (PND) 21 the offspring were separated from the mothers. One or two female offspring from the same litter were randomly selected for inclusion in the experimental groups. The mice were housed in groups of 3–4 animals per cage and the social groups were preserved until sacrifice. All animals were kept under standard laboratory conditions (21 °C, 12 h light-dark cycle with a light phase between 6.00 and 18.00) with free access to food and water. The offspring were sacrificed at the age of 13 ($n = 5$) or 17 ($n = 5$) months. The femurs and tibias were dissected and stored in microcentrifuge tubes in 1× PBS at −20 °C.

2.2. Micro-CT scanning

MicroCT imaging analyses followed published guidelines (Bouxsein et al., 2010). Prior to scanning the bone specimens were allowed to thaw at +8 °C in PBS for 12 h. The bone was wrapped in a PBS-moistened tissue paper and inserted into a plastic tube, with the proximal end pointing upwards. This container was then placed into the chamber of the μCT device (SkyScan 1174, Bruker MicroCT, Kontich, Belgium).

Projection images were acquired by scanning each bone with an image pixel size of 6.73 μm. X-rays were generated with a voltage of 50 kV and filtered with a 0.5 mm aluminum filter to reduce the beam hardening effect. One projection was collected every 0.5° over 360° rotation with an exposure time of 4000 ms. All image processing was performed with software provided by the manufacturer. The regions of interest (ROIs) were drawn for cortical and trabecular bone. For both femur and tibia the growth plate in the knee was used as a reference. To generate volumes of interest ROIs were drawn over 3.365 mm and 1.346 mm for cortical and trabecular bone, respectively. ROI drawings were started at 0.1346 mm and 1.685 mm for trabecular and cortical bone, respectively. In order to analyze the morphology of both bone compartments, the threshold was optimized individually for trabecular and cortical bone. In cortical bone analysis the 3D shrink-wrap function was used to capture both peri- and endosteal surfaces. For bone mineral density (BMD) and tissue mineral density (TMD) measurements, calcium hydroxyapatite phantom rods with a diameter of 2.0 mm and densities of 0.25 and 0.75 g/cm^3 were scanned and reconstructed to calibrate grey scale images to density.

2.3. 3-point bending

After μCT imaging the biomechanical properties of the bones were measured by the 3-point bending method using an electromechanical loading system (Instron 3366, Instron Corp., Norwood, MA, USA) controlled with Bluehill 2 (version 2.6). The biomechanical testing of midshafts of femurs and tibias was conducted as explained in a study by Jämsä et al. (2001) with a span length of 5.5 mm. The axial loading of the femoral neck was done as described in a study by Peng et al. (1999). The velocity of the load cell was 0.155 mm/s for all the loadings. Biomechanical parameters were then acquired from the load-deformation curves; stiffness was defined as the slope of the linear part of the curve, breaking force as the maximum force (Fmax) in the curve and toughness as the area under the curve from the origin to the point of fracture.