

Institute of Environmental Medicine
Environmental Health Risk Assessment

Studies On Gender-Specific Disruption Of Bone Tissue Homeostasis By Dioxins

Carolina Wejheden



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To my family

ABSTRACT

Dioxins are widespread environmental pollutants, known to cause immunosuppression, developmental and reproductive defects, as well as cancer. The toxic effects of dioxins are mediated by the aryl hydrocarbon receptor (AhR, also referred to as the dioxin receptor). Dioxins display endocrine disrupting properties and especially disturbances of the estrogen signaling system has been reported. Effects of dioxins on the estrogen system have been observed at several levels, e.g., increased metabolism of estrogen and interactions of the AhR with signaling of the estrogen receptors. As a result of the endocrine disrupting properties, dioxins might cause different responses in females and males. Bone is a dynamic tissue highly regulated by numerous factors, where estrogen is one of the key players. Bone loss is a well known effect of estrogen deficiency and can lead to osteoporosis, e.g., in post-menopausal women. A few studies have shown that dioxins interfere with bone tissue, however the mechanisms remain unknown. Moreover, no studies have been performed regarding potentially gender-related effects of dioxins in adult bone tissue.

Humans are continuously exposed to low levels of dioxins from early embryonic development throughout life. Therefore, we studied a transgenic mouse with a constitutively active AhR (CA-AhR). The bone phenotype of the female CA-AhR mice displayed loss of bone tissue, which was primarily due to an increased bone resorption. The bones in females also became softer, which might indicate an altered mineralization. The bones of CA-AhR males were on the other hand largely unaffected. However, male rats exposed to a single high dose of TCDD displayed alterations in the trabecular bone tissue already after five days exposure, indicating a responsiveness of bone tissue of either gender towards dioxins.

Gender-specific responses were also observed in differentiation cultures of osteoclasts derived from bone marrow cells of transgenic mice. Consistent with the *in vivo* results, bone marrow cultures from CA-AhR females displayed an elevated osteoclast formation. In contrast, osteoclast differentiation was inhibited in cultures from the transgenic males. At variance to the CA-AhR females, osteoclast differentiation in cultures from female wild type mice exposed to dioxin, did not display an increased osteoclast differentiation, which might be due to differences in the mode of AhR activation. The CA-AhR represents a chronic exposure situation, mimicking a long term, low dose exposure, which the cells might have adapted to, while the TCDD exposure is more acute and possible inducing additional effects. Osteoclast differentiation in the cultures from the male wild type mice exposed to TCDD responded similarly to the osteoclasts derived from CA-AhR, i.e. inhibition of osteoclast formation.

The results in this thesis show that bone is a sensitive target for dioxin exposure, leading to rapid alterations in trabecular bone tissue and gene expression in bone cells. Moreover, females seem to be more severely affected by a continuously active AhR than males. Specifically, the resorption of bone mediated by osteoclasts was altered in a gender specific manner by chronic activation of the AhR. Thus, continuous exposure to low levels of AhR ligands, such as dioxins and related environmental toxins, might be one part of the explanation for the increased incidence of osteoporosis in aging women.

LIST OF PUBLICATIONS

The present thesis is based on the four papers listed below and they will be referred to in the text by their roman numerals.

- I. **Carolina Wejheden**, Sara Brunnberg, Annika Hanberg and P. Monica Lind. Osteopontin: A rapid and sensitive response to dioxin exposure in the osteoblastic cell line UMR-106. *Biochemical and Biophysical Research Communication* 341 (2006) 116-120.
- II. P. Monica Lind, **Carolina Wejheden**, Rebecca Lundberg, Pedro Alvarez-Lloret, Sanne A.B. Hermesen, Alejandro B. Rodriguez-Navarro, Sune Larsson and Agneta Rannug. Short-term exposure to dioxin impairs bone tissue in male rats. *Chemosphere* 75 (2009) 680-684.
- III. **Carolina Wejheden**, Sara Brunnberg, Sune Larsson, P. Monica Lind, Göran Andersson* and Annika Hanberg*. Transgenic mice with a constitutively active aryl hydrocarbon receptor (CA-AhR) display a gender specific bone phenotype. *Toxicological Sciences*, 2009, *in press*
- IV. **Carolina Wejheden**, P. Monica Lind, Annika Hanberg and Göran Andersson. Osteoclastogenesis is disturbed differently by AhR activation in osteoclast differentiation cultures from male and female mice. *Manuscript*

* These authors contributed equally to this study

Additional publications not included in the thesis

Rebecca Lundberg, Björn Munro Jenssen, Àngels Leiva-Presa, Monika Rönn, Carolina Hernhag, **Carolina Wejheden**, Sune Larsson, Jan Örberg, P. Monica Lind. Effects of short-term exposure to the DDT metabolite p,p'-DDE on bone tissue in male common frog (*Rana Temporaria*). *Journal of Toxicology and Environmental Health, Part A*. 2007;70(7): 614-619.

Glen A. Fox, Rebecca Lundberg, **Carolina Wejheden**, Lars Lind, Sune Larsson, Jan Örberg J, P. Monica Lind. Health of herring gulls (*Larus argentatus*) in relation to breeding location in the early 1990s. III. Effects on the bone tissue. *Journal of Toxicology and Environmental Health, Part A*. 2008;71(21):1448-1456.

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LIST OF ABBREVIATIONS

3-MC	3-Methylcholantrene
AhR	Aryl hydrocarbon receptor
ALP	Alkaline phosphatase
Arnt	Aryl hydrocarbon receptor nuclear translocator
AHRR	Aryl hydrocarbon receptor repressor
bHLH-PAS	Basic helix-loop-helix Per-Arnt-Sim
BMC	Bone mineral content
BMD	Bone mineral density
BW	Body weight
CA-AhR	Constitutively active aryl hydrocarbon receptor
CSA	Cross sectional area
CTX	C-terminal telopeptide of collagen type I
CYP	Cytochrome P450
DRE	Dioxin responsive element
ERs	Estrogen receptors (α and β)
ERK	Extracellular signal-regulated kinase
HIF-1 α	Hypoxia inducible factor 1 α
Hsp90	Heat shock protein 90
MAPK	Mitogen-activated protein kinases
MMP	Matrix metalloproteinase
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-para-dioxins
PCDFs	Polychlorinated dibenzofurans
PCR	Polymerase chain reaction
PINP	N-terminal propeptide of type I procollagen
pQCT	Peripheral quantitative computed tomography
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RANK	Receptor activator of nuclear factor- κ B
RANKL	Receptor activator of nuclear factor- κ B ligand
RT-PCR	Reverse transcriptase polymerase chain reaction
TBV	Trabecular bone volume
TCDD	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin
TEF	Toxic equivalency factor
TEQ	Toxic equivalence
TRAP	Tartrate resistant acid phosphatase
XAP2	Hepatitis B virus X-associated protein 2
XRE	Xenobiotic responsive element

INTRODUCTION

DIOXIN

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are persistent environmental pollutants and belong to the group of compounds more commonly referred to as dioxins. In total there are 210 congeners, 75 dioxins and 135 furans, but if the dioxin-like polychlorinated biphenyls (PCBs) are included the number of congeners increases [1-4]. The dioxins and the dioxin-like PCBs share a similar structure shown in Figure 1. However, not all of the congeners are considered to be of toxic concern. The congeners considered being of toxic concern and their individual toxic equivalency factors (TEF) are listed in Table 1 [3].

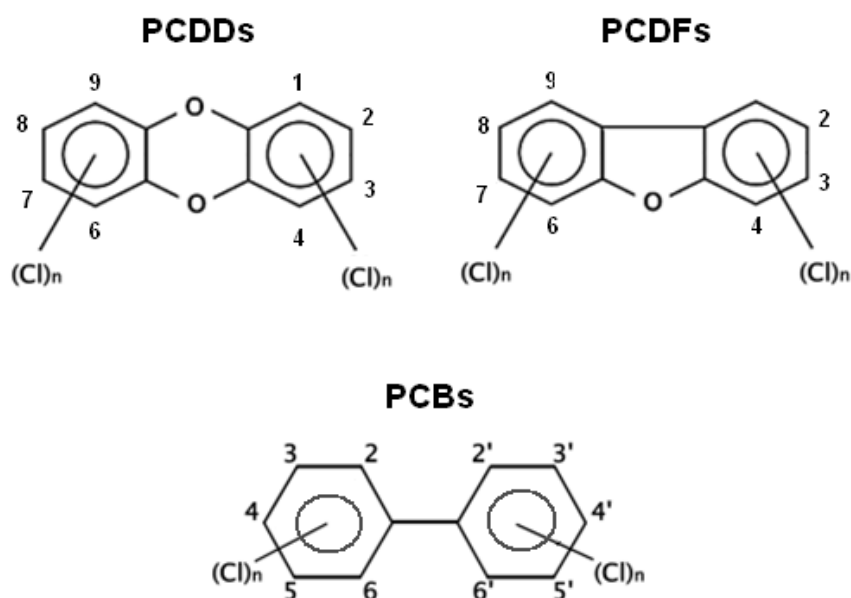


Figure 1. Molecular structure of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs).

Dioxins are known to cause a variety of adverse effects. For example, at high doses dioxins cause chloracne, ocular lesions and wasting syndrome, while the more sensitive effects include cancer, immunosuppression, reproductive toxicity and endocrine disruption. In 1997 the International Agency for Research on Cancer (IARC) concluded that TCDD is a human carcinogen [5]. The classification of TCDD as a carcinogen was initially controversial, but more recent studies supports the classification of TCDD as a human carcinogen (Group 1) [6].

Table 1. Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like PCBs considered to be of toxic concern, listed together with their respective toxic equivalency factor (TEF). The dioxin-like PCBs are listed with their IUPAC number.

Compound	TEF
<i>Polychlorinated dibenzo-p-dioxins</i>	
2,3,7,8-TCDD	1
1,2,3,7,8-PeCDD	1
1,2,3,4,7,8-HxCDD	0.1
1,2,3,6,7,8-HxCDD	0.1
1,2,3,7,8,9-HxCDD	0.1
1,2,3,4,6,7,8-HpCDD	0.01
OCDD	0.0003
<i>Polychlorinated dibenzofurans</i>	
2,3,7,8-TCDF	0.1
1,2,3,7,8-PeCDF	0.03
2,3,4,7,8-PeCDF	0.3
1,2,3,4,7,8-HxCDF	0.1
1,2,3,6,7,8-HxCDF	0.1
1,2,3,7,8,9-HxCDF	0.1
2,3,4,6,7,8-HxCDF	0.1
1,2,3,4,6,7,8-HpCDF	0.01
1,2,3,4,7,8,9-HpCDF	0.01
OCDF	0.0003
<i>Dioxin-like PCBs</i>	
PCB 77	0.0001
PCB 81	0.0003
PCB 126	0.1
PCB 169	0.03
PCB 105	0.00003
PCB 114	0.00003
PCB 118	0.00003
PCB 123	0.00003
PCB 156	0.00003
PCB 157	0.00003
PCB 167	0.00003
PCB 189	0.00003

One of the main problems with the dioxins is their resistance to metabolism. The half-life of TCDD in humans is estimated to ~7.5 years, but varies from 5.1 to 11.3 years [1, 5]. There are a few congeners with longer half-life (e.g., 1,2,3,7,8-PeCDD) than TCDD, however most of the other congeners have a shorter half-life [1]. The half-life is also dependent on dose, body composition, age and sex [1, 4]. Dioxins are fat soluble compounds that bioaccumulate in organisms, especially in adipose tissue, and are, as a consequence of their resistance to degradation and lipophilicity, biomagnified within the food-chain. Although dioxins never have been intentionally produced they are formed during incineration processes and as byproducts in industrial processes such as chlorine bleaching of paper pulp, smelting and manufacturing of herbicides and pesticides [7, 8]. This was the case with the well known herbicide called Agent Orange, which was extensively used as a defoliant during the Vietnam War. Dioxins are also formed naturally in a very small scale during volcano eruptions and forest fires [9]. Some incidents with dioxins are reviewed in Box 1.

Humans are primarily exposed to dioxins through food, which contributes with over 90% of the dioxin exposure. Highest levels of dioxins are found in meat, chicken, egg, fish, and dairy products [10]. The risk assessment of dioxins (reviewed in Box 2) concluded that the safety margin for dioxin exposure is minimal or non-existing, which means that subgroups in the population might be exposed to considerably higher concentrations. Moreover, there are risk groups which are more vulnerable to dioxin exposure, e.g. fetuses and infants. Moreover, breast fed infants have an intake of dioxins that is 1-2 orders of magnitude higher than adults [1, 2].

Accidental eruptions of dioxins, such as the Seveso accident, have given us some clues to the long term effects of dioxin exposure in humans. Nine months after the Seveso accident there were considerably more girls being born to parents exposed to TCDD, indicating a shift in the sex ratio [11]. Children to dioxin-exposed mothers displayed low birth weights, developmental delays, behavioral disorders, hearing loss as well as alterations in sexual development [12, 13]. Also certain types of cancer have been linked to dioxin exposure, such as multiple myeloma [14]. A 20 years follow-up study from the Seveso accident indicate an increased risk of developing cancer in the digestive tract (males in the rectum and females in the biliary tract) and in the respiratory tract (lung cancer in males) [15].

Box 1 – Incidents with dioxins

- 2008 – *Naples, Italy*: Mozzarella cheese, made from buffalo milk, was contaminated with high levels of dioxins.
- 2007 – *India*: High levels of dioxins were detected in the food additive called guar gum, a thickener in e.g. meat, dairy and desserts. The guar gum was contaminated with the pesticide pentachlorophenol (PCF), which contained dioxins [1].
- 2006 – *Netherlands*: Fat, used in the production of animal feed, was contaminated with dioxins, leading to polluted animal feed [1].
- 2004 – *Netherlands*: High levels of dioxins were detected in milk, which was traced to contaminated clay used in the production of animal feed [1].
- 2004 – *Ukraine*: During the election in 2004 Viktor Yushchenko, president of Ukraine, was poisoned with dioxin and his face was disfigured with chloracne [2].
- 1999 – *Belgium*: High levels of dioxins were discovered in eggs and poultry. Animal feed had been contaminated with illegally disposed PCB-based waste industrial oil. Subsequently, high dioxin levels were detected in animal based food in several other countries [1, 3].
- 1998 – *Brazil*: Citrus pulp pellets used as animal feed were contaminated with dioxins [1].
- 1997 – *USA*: Dioxin contaminated bentonite clay, used in the production of animal feed, lead to high levels of dioxin in chickens, eggs and catfish [1].
- 1990s – *Scandinavia*: High levels of dioxin were discovered in fish from the Baltic Sea [4]. High dietary consumption of fish from the Baltic Sea was associated with low birth weight a Swedish cohort [5].
- 1979 – *Taiwan*: Rice oil, used in cooking, was contaminated with PCBs, PCDFs and PCDDs, resulting in the poisoning of over 2000 people (Yu-Cheng-disease) [6].
- 1976 – *Seveso, Italy*: An explosion in a chemical factory released more than 30 kg TCDD into the environment and contaminated 25 km² [7].
- 1968 – *Japan*: In Kyusho, in Japan, a strange skin disease appeared, the Yusho-disease. This skin lesion was chloracne caused by PCB and PCDF contaminated rice oil used in cooking [6].
- 1960s – *Vietnam*: US Air Force used the heavily dioxin-contaminated Agent Orange as a defoliant during the Vietnam War. It is estimated that 3000 villages and at least 2.1 million people were sprayed directly with Agent Orange [8].

- [1] WHO, Dioxins and their effects on human health, Fact sheet N°225, 2007.
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The aryl hydrocarbon receptor - AhR

Dioxins and related aryl hydrocarbons bind to the aryl hydrocarbon receptor (AhR) and most, if not all, toxic effects caused by dioxins are mediated by binding and activating this receptor [16]. The AhR belongs to the family of basic helix-loop-helix (bHLH)/PER-ARNT-SIM homology transcription factors [17]. AhR resides in the cytosol together with two molecules of Hsp90, p23 and the immunophilin-like protein XAP2 (Figure 2). Upon ligand binding the complex dissociates and the receptor undergoes a conformational change that exposes a nuclear localization signal (NLS). The ligand-bound AhR is transported into the nucleus where it forms a heterodimer with its partner protein AhR nuclear translocator (Arnt, also known as hypoxia inducible factor-1 β (HIF-1 β)). This heterodimer binds to specific regions in DNA called xenobiotic responsive elements (XREs or dioxin responsive elements; DREs) [17]. Activation of the AhR will result in induction or repression of diverse array of genes. The most well studied gene for AhR activation is cytochrome P450 1A1 (CYP1A1) and the most potent inducer of CYP1A1 is TCDD [18]. CYP1A1 metabolizes endogenous substrates such as arachidonic acid (AA), bilirubin and estrogen [19], but also exogenous compounds such as caffeine, flunarizine (a selective calcium-entry blocker) and R-warfarin [20].

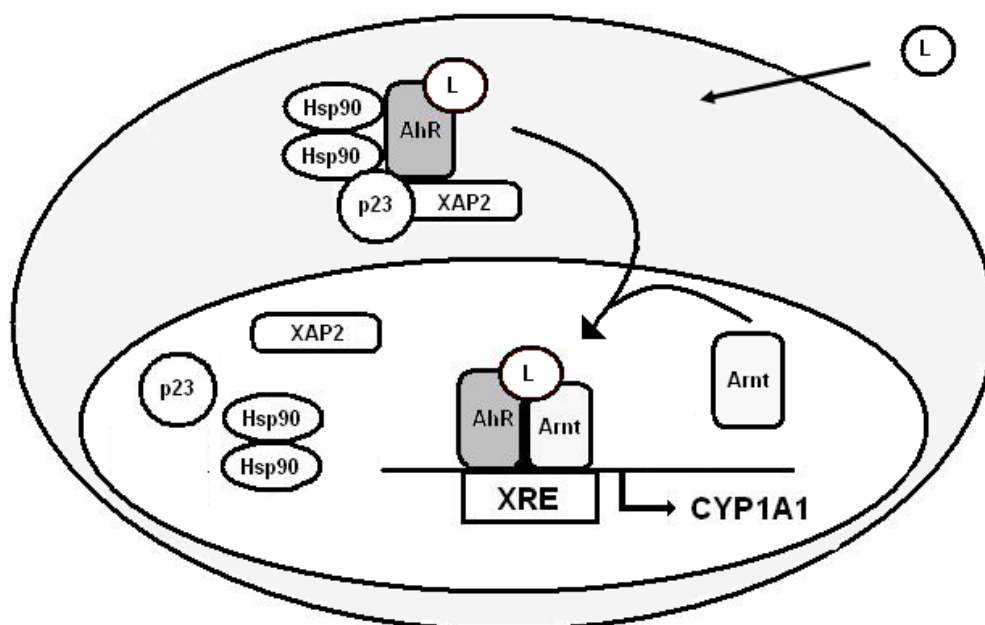


Figure 2. Simplified pathway of AhR signaling. The AhR resides in the cytosol with 2 Hsp90, XAP2 and p23. Upon ligand binding of the AhR, the complex dissociates, translocates into the nucleus and AhR forms a heterodimer with Arnt. The AhR/Arnt-dimer binds XRE in the DNA and modulates the transcription of various genes, e.g., induction of CYP1A1 is a widely accepted marker for AhR activity.

AhR diversity

AhR is a well conserved protein found in vertebrates and invertebrates. The earliest homologs of the receptor are found in metazoans. In vertebrates there are at least three members in the AhR gene family, AhR1, AhR2 and AhR repressor (AhRR). Mammals however, have only one of the AhRs [21]. Fish have both AhR genes; AhR1 and AhR2. AhR 1 is the ortholog to the mammalian AhR, however, AhR 2 is the predominant form in fish [21]. An interesting feature of the invertebrate AhRs is their inability to bind typical AhR ligands, suggesting that the role in regulating xenobiotic metabolizing enzymes evolved in vertebrates [22].

Although the physiological role of the AhR is not fully established it is suggested to be important in the development of different organs, which has been demonstrated in studies with mice lacking the AhR (AhR knock out; AhRKO). AhRKO mice display alterations in e.g. the liver [23], immune system [24], ovary [25] and heart [26], indicating the importance of a functional AhR during the development of certain organs. AhR null-mice are resistant to dioxin toxicity, implying the fundamental role of AhR in mediating the toxic effects of dioxins [27].

There is a great variety in the sensitivity towards dioxins, both between species and within the same species. Regarding acute toxicity, hamsters are among the most dioxin resistant mammals, while the guinea pig is one the most sensitive mammal known [28]. The rat strain Long-Evans (L-E) is 1000-fold more sensitive to acute dioxin exposure compared to Han/Wistar (H/W) rats. The difference between the L-E rats and the H/W rats is due to a point mutation in the resistant H/W strain resulting in an altered splicing and lack of approximately 40 amino acids in the trans-activation domain of the AhR [29]. Also mice differ in sensitivity to AhR ligands. The C57BL/6 encodes an alanine in codon 375 in the ligand binding domain, resulting in a 10 fold higher ligand binding affinity for TCDD compared to other mouse strains carrying a valine in this position, e.g. the DBA/2 mouse strain [30].

Endogenous AhR-ligands

No endogenous high affinity ligand to the AhR receptor has been identified, however there are both endogenous compounds and dietary compounds with the ability to bind the AhR. Several photooxidation products, generated by UV radiation of the amino acid tryptophan, have been shown to bind and activate the AhR with high affinity [31].

FICZ (6-formylindolo[3,2-*b*]carbazole), a product of UV radiation, was identified as an AhR ligand with high affinity for the receptor [32]. Moreover, metabolites of FICZ have recently been identified in human urine [33]. Also other potential endogenous ligands to the AhR have been suggested, e.g., metabolites of arachidonic acid (AA) and tetrapyrrols [31].

Also dietary ligands to the AhR have been identified, and these include curcumin, carotenoids and flavonoids. Although the majority of these ligands are weak agonists, some dietary indoles, such as tryptophan, can be converted into more potent AhR-ligands in the digestive tract [31].

AhR knock out mouse

Han/Wistar rats are resistant to dioxin exposure due to a deviant AhR. Similar, mice completely lacking an AhR (AhRKO) are also resistant to dioxin exposure. AhRKO mice exposed to TCDD do not display the typical toxic effects observed in wild type mice exposed to TCDD, such as wasting syndrome, thymic atrophy and teratogenic effects [27, 34-36]. However, the AhRKO mice also indicate the requirement for a functional AhR in the development of certain organs, such as the liver and immune system [23, 24]. Also, reproduction in these mice were reduced, where the female AhRKO displayed difficulties maintaining the conceptuses during pregnancy [37].

The CA-AhR mouse

In contrast to the knock out of the AhR, a model with a constitutively active AhR (CA-AhR) has been developed in our laboratory. The mutated constitutively active AhR (CA-AhR) was generated by partial deletion of the minimal ligand-binding domain of the AhR and does not require ligand for activation (Figure 3) [38].

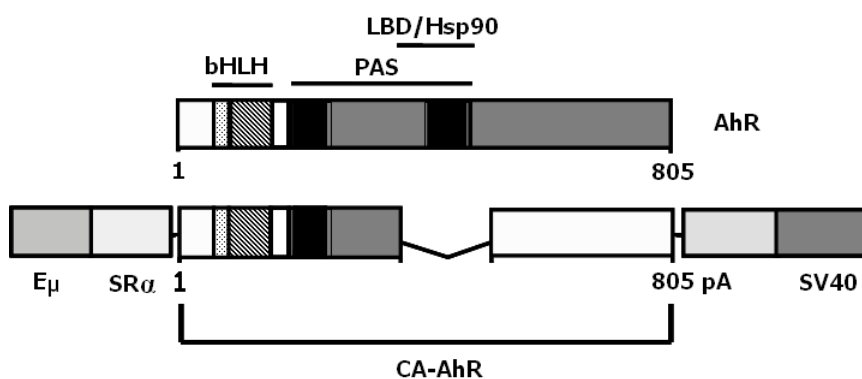


Figure 3. Structure of the wild type AhR (upper) and the construct encoding the constitutively active AhR (CA-AhR) (lower).

Previous studies of this mouse model have concluded that the activity of the mutated receptor resembles continuous exposure to low doses of dioxins. Some of the typical effects in wild type mice exposed to dioxin are also observed in these transgenic mice, e.g. CYP1A1 induction, thymus atrophy and increased liver weights [39, 40]. In transgenic mice the hepatic induction of CYP1A1 corresponds to a single dose exposure of 0.3 µg TCDD/kg bw in wild type mice [39]. Thus, the activity of CA-AhR is relatively low.

These transgenic mice display a reduced life-span, which correlated to the development of tumors in the glandular part of the stomach [39]. Interestingly, animals treated with AhR ligands have been reported to display similar gastric lesions [41, 42]. Further investigation of these gastric tumors revealed that the expression of osteopontin was reduced in the CA-AhR mice [43]. Also the immune system was affected in these transgenic mice. The CA-AhR mice display an enlarged population of mature bone marrow derived B cells [40], which also has been reported in dioxin exposed wild type mice [44]. However, the population of peritoneal B1 cells was decreased in CA-AhR mice [40]. There seem to be some gender-related alterations in certain organs of the CA-AhR mice, where the relative kidney and heart weights were increased in male CA-AhR mice but not females [45]. Also a suspected tumorigenic effect on liver was demonstrated in the male CA-AhR mice [46].

A similar transgenic mouse has been generated by Nohara *et al.* [47]. The expression of the constitutively active receptor was under the regulation of the T cell specific CD2 promoter and the CA-AhR was reported to be expressed in thymus, spleen, lung and to a minor extent in the kidney. The activity of the CA-AhR in thymus, measured by CYP1A1, corresponded to the exposure of a single dose of 20 µg TCDD/kg bw, which reflects a considerably higher activity of the CA-AhR compared to our CA-AhR mouse. The mice were fertile, displayed normal sex-ratio at birth and no increased mortality after birth. However, like the transgenic mice in our laboratory, the thymus weight was reduced in female transgenic mice [47].

TCDD cause chloracne in humans and one postulated mechanism for this effect might be an impact of TCDD on keratinocytes [48, 49]. A third transgenic mouse with a constitutively active AhR has been generated, where the construct encoding the receptor was under the regulation of the human keratin 14 (K 14) promoter and

expressed in keratinocytes. These mice develop inflammatory skin lesions and the expression of many inflammation-related genes was increased (e.g., IL-1 β and IL-18) [50]. Increased expression of IL-1 β has also been reported in two keratinocyte cell lines treated with TCDD [51].

Non-genomic effects of dioxins

Although most of the effects are thought to be mediated by the AhR, non-genomic effects have also been reported. In male rats TCDD stimulated the activity of extracellular signal-regulated kinases (ERKs) [52]. A later *in vitro* study also showed that TCDD and other AhR ligands activated the ERKs and the Jun N-kinases (JNKs) in the mitogen-activated protein kinase (MAPK) pathway [53]. After only 10 minutes TCDD caused phosphorylation of ERK1/2 and activation of JNKs. Interestingly, activation of these kinases did not require the AhR itself, since the activation of the kinases was detected in an AhR deficient cell line. Furthermore, activation of the MAPKs was crucial for the AhR-dependent transcription of the CYP1A1 gene [53].

Endocrine disruption

Dioxins are endocrine disrupting compounds and are well-known to interfere with estrogen signaling pathways. Both estrogenic and anti-estrogenic properties have been reported. Anti-estrogenic properties of dioxins are well documented and reviewed in Safe *et al.* 2003 [54]. An example is rodent uterus, where dioxin inhibited several 17 β -estradiol (E2) induced responses such as increased uterine wet weight [55], EGF receptor binding and EGF receptor mRNA [56] and c-fos mRNA levels [57]. The postulated mechanisms for the anti-estrogenic effects of dioxins are:

- ❖ Increased metabolism of E2 by CYP1A1 and CYP1B1 [58]
- ❖ Presence of inhibitory DREs (iDRE) in estrogen responsive gene promoters (e.g., cathepsin D [59] and c-fos [60])
- ❖ Competition for common nuclear co-regulatory proteins [61] and
- ❖ Degradation of estrogen receptors [62].

However, also estrogenic properties of dioxins and AhR-ligands have been reported. In estrogen deprived mouse and rat uterus, 3-methylcholantrene (3-MC) and TCDD, respectively, induced estrogenic responses [63, 64]. The ligand-activated AhR/Arnt complex directly associated with the unliganded estrogen receptor α and β (ER α and

ER β) and induced the transcription of EREs in OVX mouse uterus. However, at high doses of estrogen, the AhR-ligands displayed anti-estrogenic properties [63]. Furthermore, TCDD induced an estrogen-like response on the gene expression in uterus of ovariectomized mice [65]. One hypothesis is that dioxins and dioxin-like compounds have different effects in estrogen deprived tissues and in tissues with normal estrogen level. For example, the dioxin-like PCB 126 displayed weak estrogenic activity in bone tissue in estrogen-deprived rats, but when estrogen was present PCB 126 displayed anti-estrogenic activity [66]. Also interference with the testosterone signaling pathways has been suggested, where anti-androgenic effects were reported [67].

Health risk assessment

All dioxins are thought to mediate their toxicity through the same pathway/pathways, thus to facilitate the risk assessment the concept of toxic equivalency factors has been developed. The toxicity of each congener has been compared to the toxicity of the most toxic congener 2,3,7,8-TCDD, which has been given the value of 1. This relative value is called toxic equivalency factor (TEF). Individual TEF values together with the concentration of each congener can be used to calculate the total toxic equivalence (TEQ). The TEQ for each congener can then be added to generate the total TEQ value for all congeners in the mixture.

$$\text{TEQ} = (\text{PCDD}_a \times \text{TEF}_a) + (\text{PCDF}_b \times \text{TEF}_b) + (\text{PCB}_c \times \text{TEF}_c)$$

Several studies have estimated that humans in industrialized countries have a daily intake of 1-3 pg TEQs/kg body weight. The risk assessment performed by EU concludes that the tolerable daily intake (TDI) is 2 pg TEQ/kg body weight [1, 2]. Thus the safety margin for human exposure to dioxins is minimal or non-existing. An overview of the risk assessment for dioxin is shown in Box 2.

Box 2 – Health risk assessment of dioxins

Risk assessments are based on either a no-observed-adverse-effect-level (NOAEL) or a lowest-observed-adverse-effect-level (LOAEL), which is extrapolated to a NOAEL using an uncertainty factor. Uncertainty factors are also used to extrapolate differences between humans and experimental animals and differences in susceptibilities within the human population.

Although data from human exposure were evaluated, the risk assessment of dioxins was based on animal studies, where the most sensitive adverse effects following TCDD exposure were developmental effects in female and male rats. In the case of dioxins, body burdens were used to scale the doses between species, thus no interspecies uncertainty factor was necessary. To account for the difference in susceptibility among humans only a small uncertainty factor was needed. A total uncertainty factor of 10 for the extrapolation was used to calculate the tolerable daily intake (TDI), which ended up at 2 pg TEQ/kg body weight [1, 2].

The estimated average intake of dioxins for adult humans is 2 pg TEQ/kg body weight/day [3], while the intake of breast-fed infants, on a body weight basis, has been estimated to be 1-2 orders of magnitude greater than for adults [4]. The concentrations of PCDDs, PCDFs and dioxin-like PCBs in breast milk have however decreased up to 50% in industrialized countries during the last 10 years before the risk assessment was performed.

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BONE TISSUE

Bone tissue is a highly dynamic tissue, which is constantly undergoing catabolic and anabolic processes to maintain flexibility and adapt to new demands from the environment. It has been estimated that it takes 10 years to completely renew a human skeleton [68]. An imbalance either in bone formation or bone resorption can result in bone disorders such as osteoporosis and osteopetrosis.

Structurally bone can be divided into two different types: cortical and trabecular bone tissue. Cortical bone is a compact structure mainly located at the mid shaft of long bones but also comprise the outer shell of bones. Cortical bone has a slow metabolic rate and approximately 4% is renewed every year [68, 69]. Trabecular bone tissue is a spongy network of trabeculae located inside the cortical shell at the ends of the long bones. The metabolic activity of trabecular sites is higher than the metabolic rate in cortical bone and roughly 28% is renewed every year. Our skeleton has three main functions: to protect vital organs, provide support to our extremities and to act as a mineral reservoir for phosphate and calcium [68, 69].

There are four major cell types in bone tissue: the osteoblasts, the osteocytes, the lining cells and the osteoclasts. The osteoblasts produce bone matrix and then become encapsulated in the matrix and differentiate further into osteocytes, while the osteoclasts resorb bone tissue. During normal conditions, bone formation equals bone resorption resulting in a balanced bone remodeling. However, when the balance is shifted, in either direction, different pathological conditions can be manifested, such as osteoporosis (loss of bone tissue) or osteopetrosis (excess of bone tissue).

Osteoblasts

The anabolic process of bone formation is performed by the osteoblasts. Osteoblasts originate from mesenchymal stem cells (MSC) and a crucial transcription factor for osteoblast commitment is Runx2 (also known as Cbfa1). Runx 2 is the earliest differentiation marker for osteoblasts currently known and regulates the expression of osteoblast genes e.g. type I collagen alpha chain, (Col I) osteopontin (OPN), bone sialoprotein (BSP), osteocalcin (OCN) and alkaline phosphatase (ALP) [70, 71]. It is now widely accepted that Runx 2 is the master gene of osteoblast differentiation [72]. The expression of Runx 2 is regulated by Twist-1, which inhibits Runx 2, and Msx2 and Bapx1, which both induce Runx 2. Osterix is a Runx 2-dependent transcription

factor required for osteoblast differentiation. While Runx 2 also is involved in chondrocyte differentiation, Osterix specifically induces osteoblast differentiation [72]. The most abundant product from osteoblast activity is collagen type I. About 90% of the organic bone matrix is collagen type I, but collagen type V is also present [73]. A simplified overview of osteoblast differentiation and fate is shown in Figure 4. The bones become hard when the collagen fibrils are deposited with hydroxyapatite crystals, a process called mineralization (see “Inorganic bone matrix”)

Osteocytes

Osteocytes are by far the most abundant cell type in bone tissue and constitute over 90% of the total number of cells in bone [74]. Osteocytes are further differentiated osteoblasts, which have become encapsulated in the mineralized bone matrix. When the bone forming process has come to an end the osteoblasts have four options:

- ❖ differentiate to osteocytes,
- ❖ transform into lining cells (see next section)
- ❖ enter apoptosis
- ❖ trans-differentiate into chondrocytes [74].

The osteocytes are connected to one another via long cell extensions and communicate through gap junctions. The life-span of osteocytes varies depending on the remodeling rate of the particular bone. If the osteocyte resides in bone with a slow turnover rate, the half-life may be decades [75].

The exact role of the osteocytes is still not entirely known, however they are thought to function as mechanosensory cells. Their long cell-extensions, connected with gap-junctions to other osteocytes, lining cells and osteoblasts, enable them to rapidly transmit signals. Osteocytes can produce signals that induce or inhibit bone resorption as well as factors that inhibit bone formation, e.g. sclerostin [76]. Osteocytes are also suggested to have a central role in the mineralization of bone tissue and in the total systemic phosphate regulation [77].

Lining cells

Like the osteocytes, lining cells are terminally differentiated osteoblasts that cover the surface of bone, connected to one another through gap junctions. The exact roles of the

lining cells are still not entirely clear. However, they are suggested to prepare the bone surface for the osteoclasts by digesting the thin layer of unmineralized osteoid located on the bone surface under the lining cells, thereby exposing the bone surface to the osteoclasts. The lining cells are also suggested to produce and secrete chemokines that recruit osteoclast precursors [78, 79].

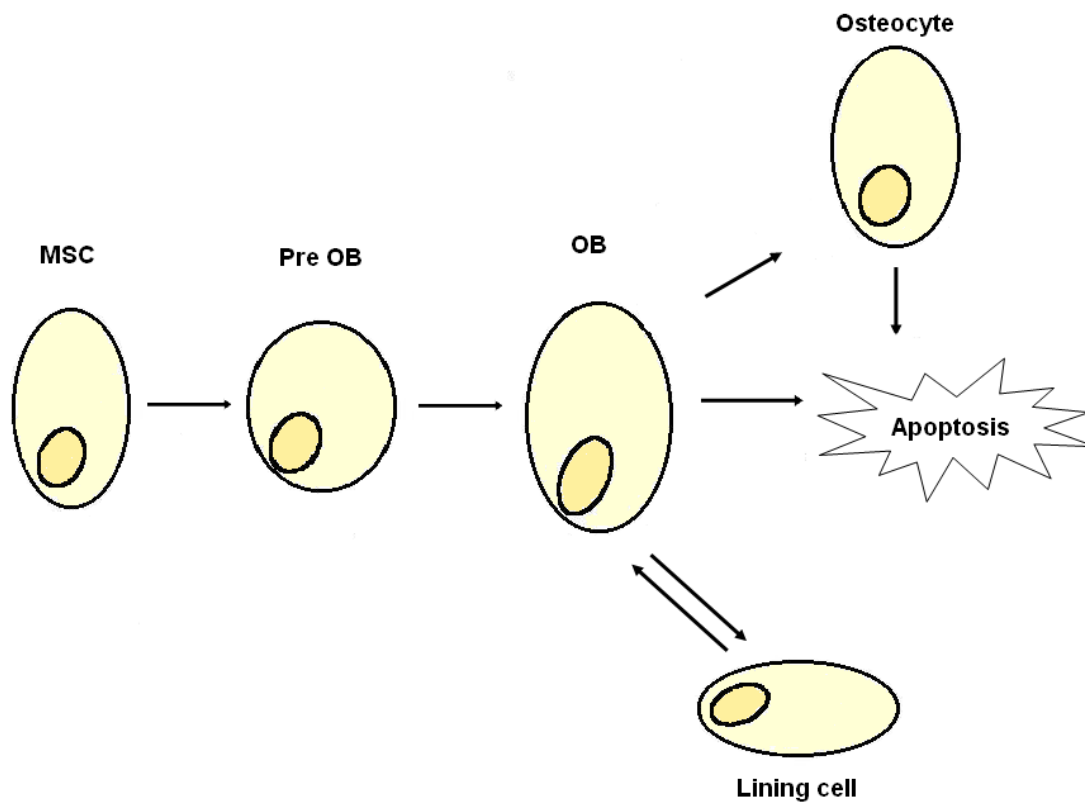


Figure 4. Overview of osteoblast differentiation from mesenchymal stem cells (MSC) to pre osteoblasts (Pre OB) and then into mature osteoblasts (OB). The main products by the mature OB are collagen type I, osteopontin, alkaline phosphatase and osteocalcin.

Osteoclasts

Bone resorption is performed by multinucleated osteoclasts, which are ultimately derived from pluripotent hematopoietic stem cells (HSC). The earliest hematopoietic precursor that can form osteoclasts is the CFU-GM, which besides osteoclasts also can differentiate into monocytes/macrophages. Macrophage colony stimulating factor (M-CSF) is an essential cytokine for osteoclastogenesis that stimulates proliferation and prevents apoptosis of osteoclast precursors [80, 81]. The most important signal for osteoclast differentiation is the interaction of receptor activator of nuclear factor (NF)- κ B ligand (RANKL), produced by osteoblasts in the periosteum and stromal cells in bone marrow, with the osteoclast receptor RANK. The crucial roles of RANKL and RANK are demonstrated in mice deficient of either RANKL or RANK where

osteoclasts are absent. Binding of RANKL to RANK induces the transcription of NFATc1, which turns on an autoregulatory loop. Osteoprotegerin (OPG) is a decoy receptor for RANKL and can inhibit the RANKL-RANK interaction, thereby inhibiting osteoclastogenesis [80-82]. A simplified figure of osteoclast differentiation is shown in Figure 5.

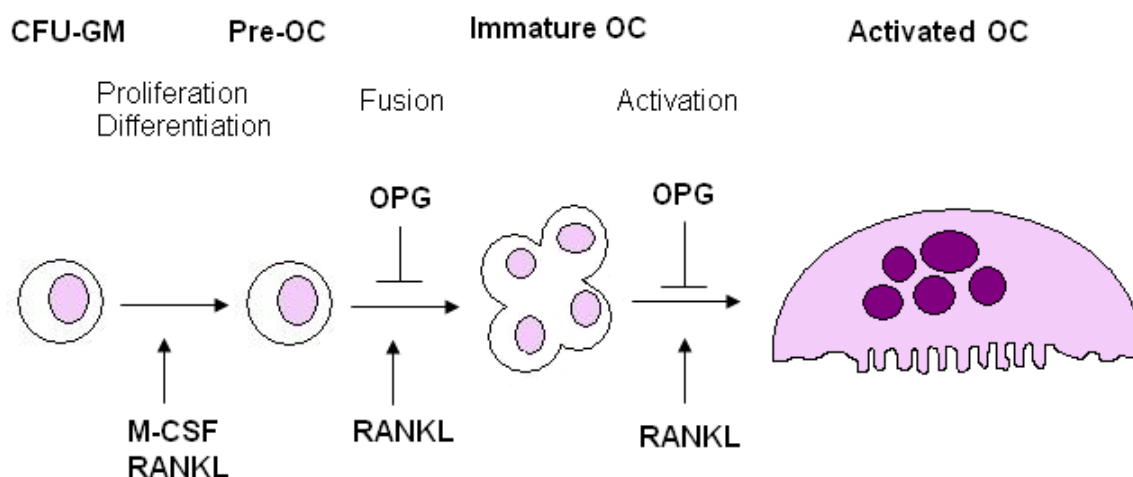


Figure 5. Simplified picture of osteoclast differentiation from HSC, where the earliest osteoclast precursor is CFU-GM. RANKL is the most important factor for osteoclast differentiation and can stimulate osteoclast differentiation at several steps

Interestingly, AhR has been suggested to be involved in the regulation of HSC. The exact role is not known, but AhR is suggested to regulate the balance between quiescence and proliferation in the HSC population [83]. Thus, TCDD might influence the precursor population of osteoclasts.

The mature and resorbing osteoclast is highly polarized with specialized membrane domains, shown in Figure 6. The process of bone degradation is specifically located to the resorption lacunae (RL) and is performed by the ruffled border membrane (RB). To demineralize the bone tissue, osteoclasts must retain a low pH value in the resorption lacunae, this is performed with a V-type ATPase proton pump and chloride channel 7 (CCl7) situated in the ruffled border. The organic bone matrix is degraded by lysosomal cysteine proteinases, primarily cathepsin K, and matrix metalloproteinases (MMPs), such as MMP-9, secreted into the resorption lacunae. Free oxygen radicals may also contribute to extracellular matrix degradation [84, 85]. To maintain this acidic and rather harsh environment in the resorption lacuna, the membrane of the osteoclast is

tightly attached to the bone in the structure called the sealing zone (SZ). One plausible mechanism for this is the binding of matrix proteins on the bone surface, such as osteopontin, to the integrin $\alpha_v\beta_3$ on osteoclasts. Also the binding of collagen type I to $\alpha_2\beta_1$ and fibronectin to $\alpha_5\beta_1$ are important in the SZ. The waste products, generated in the resorption lacunae, are transported in vesicles from the ruffled border to the functional secretory domain (FSD), where they are excreted [84, 86].

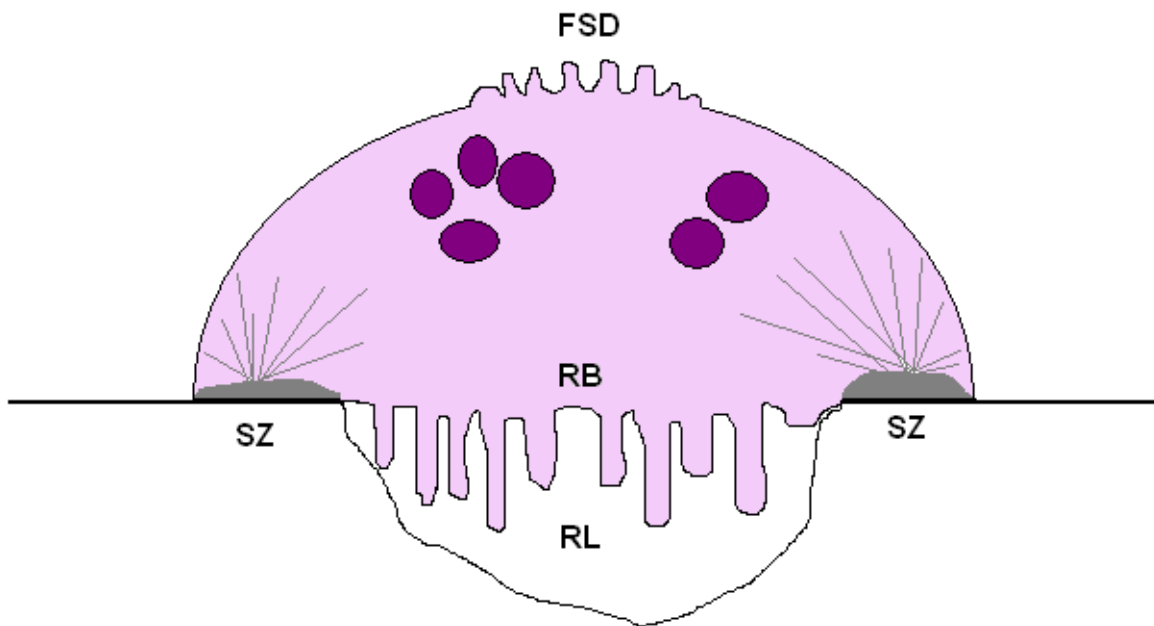


Figure 6. During bone resorption the osteoclast is highly polarized with specific membrane domains; FSD, functional secretory domain; RB, ruffled border; RL, resorption lacunae and SZ, sealing zone.

Bone matrix

The extracellular bone matrix consists of one organic part and one inorganic part. The osteoblasts produce the organic bone matrix, which primarily consists of collagen type I, but also collagen type V [87]. The osteoblasts also produce noncollagenous protein such as osteopontin (OPN), osteocalcin, fibronectin, osteonectin and alkaline phosphatase (ALP) [88]. These proteins are secreted by the osteoblasts and together with collagen fibers assemble extracellularly as osteoid matrix. This organic matrix is subsequently converted into hard mineralized bone tissue by deposition of hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3(\text{OH})$) crystals on the collagen fibers. Hydroxyapatite is the major constituent of bone tissue and contributes to 70% of the total bone mass. The noncollagenous proteins, such as osteocalcin and osteopontin are thought to participate

in the regulation of the amount and size of the hydroxyapatite crystals. The organic matrix contribute to elasticity and flexibility of the bones, while the inorganic matrix provides the strength and mechanical rigidity [89]. Structures similar to bone or that appears to be bone-like are listed in Box 3.

Box 3. Structures resembling bone

Antlers

Antlers are the bony appendages carried by many deer species, such as reindeer and moose and are the only mammalian bone that is capable of regeneration. In addition, antlers are among the fastest growing organs in the animal kingdom, with a maximum growth rate of 4 cm/day. Antlers consist of bone tissue, although there are differences compared to the bones in the skeleton [1].

Dental tissue

Similar to bone, dental tissue is also a calcified tissue. The teeth are formed by dentine, which largely consists of collagen produced by cells called odontoblasts. These cells share the same origin as the osteoblasts and the differentiation is very similar, e.g. Runx 2 controls the differentiation of both osteoblasts and odontoblasts[2]. The cells that resorb tooth tissue are called odontoclasts and they are almost identical to osteoclasts [3]. Thus dental tissue and bone tissue are closely related.

Ivory

Ivory is used in ornaments and had many practical uses before the plastic era e.g., piano keys and Scottish bagpipes. Elephants have been, and still are despite the worldwide ivory trade ban in 1989, extensively hunted for their tusks. Also other animals have/had ivory in teeth and tusks, e.g. hippopotamus, walrus, mammoth and narwhal. Ivory is formed in the same way as dental tissue and therefore resembles bone tissue to a great extent [4].

Rhino horn

Rhinos have almost become extinct as a result of hunting. They were hunted because their horns were believed to possess medical properties and to functions as an aphrodisiac. Trade with rhino horns is illegal since 1993, but poaching occurs. Rhino horn is still used in traditional Asian medicine and knife handles in Yemen [5]. Rhino horns consist of compressed keratine fibers, lack a bone core and is not attached to the skull. Thus, despite the appearance, rhino horn cannot be classified as a bony structure. It is more closely related to hair and nails. [6].

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BONE TISSUE AND DIOXINS

Although bone tissue toxicology is a rather new field of research, a growing number of studies suggest that dioxins and other synthetic AhR-ligands disturb bone tissue homeostasis.

In vivo

An *in vivo* study with two different rat strains, with different sensitivity to dioxins, showed the importance of a functional AhR in dioxin mediated bone toxicity [90]. Han/Wistar (H/W) rat is less sensitive to dioxin due to a deviant AhR, while the Long-Evans (L-E) rat with a normal AhR is more susceptible to dioxins [29, 91]. In the diaphysis, female L-E rats displayed reduced tibial length decreased bone cross sectional size and cortical area at a dose of 1.7 μg TCDD/kg bw. Moreover, the mechanical properties of the bones in female L-E rats were altered at 17 μg TCDD/kg bw. The breaking force and the stiffness of the bones were reduced, indicating weaker and softer bones. Also the trabecular bone tissue was affected, where the trabecular bone volume (TBV) was decreased at 17 μg TCDD/kg bw [90].

However, despite the deviant AhR in the H/W rats, TCDD caused changes in the bones of female H/W rats, although at higher doses. Similar responses to the female L-E rats were observed in diaphysis of the female H/W rats exposed to 170 μg TCDD/kg bw. However, no effect was detected on the TBV [90]. This study clearly demonstrated the central role of AhR in TCDD toxicity in bone tissue. The higher doses required to induce effects in the female H/W rats are most likely due to the less efficient AhR, however, its is intriguing to speculate if non-genomic effects of TCDD can contribute to these effects.

In utero and lactational exposure to TCDD caused effects on developing bone tissue in a dioxin sensitive rat line [92]. By crossing H/W rats with L-E rats, three different rat lines (A, B and C) were generated and they displayed different sensitivity to short-term toxic effects of TCDD. Line A was most resistant, line B was less resistant than A, but not as sensitive as line C. Three different studies were performed, a dose-response study, a timing study and a one-year follow-up study. Bone changes were only seen in line C at a maternal dose of 1 μg TCDD/kg bw in the dose response study. Offspring to rats exposed to a single dose of 1 μg TCDD/kg bw displayed a reduced cortical bone mineral density (BMD) in tibia and reduced cross sectional area (CSA), periosteal

circumference, endosteal circumference and cortical BMD in femur [92]. In the timing study, both male and female offspring displayed similar changes when the mothers were exposed during gestation, postnatally or during lactation.

Female rhesus monkeys exposed to TCDD *in utero* and through lactation (maternal dose of 40.5-42 ng TCDD/kg bw on gestational day 20) displayed increased total bone mineral content (BMC), CSA and periosteal circumference in the femoral diaphysis on post natal day 90 [93]. However, no alterations were detected in the diaphysis of male monkeys. Also the metaphysis was affected by TCDD in female monkeys, where the trabecular BMC increased dramatically. No changes were detected in the male monkeys. Interestingly, the physical properties of the bones from female monkeys were not changed by TCDD, while bones from male monkeys exposed to TCDD (maternal dose of 40.5-42 ng TCDD/kg bw) displayed a decreased displacement at failure [93]. Thus, although the geometrical dimensions and content in femurs of female monkeys were affected this was not reflected in the strength of the bones. The decreased displacement in the male monkeys might reflect changes in qualitative parameters that cannot be detected by pQCT or three-point bending. It should, however, be noted that the TCDD exposed groups in this study were quite small: 6 males and only 3 females [93].

Male mice exposed to TCDD via lactation (maternal dose of 15 µg TCDD/kg bw) displayed a reduced amount of mineralized bone in the tibial diaphysis on post natal day 21 [94]. Also the metaphysis displayed a reduced amount of mineralized bone, while there was an increase in the unmineralized osteoid. The mRNA expression and serum levels of bone formation markers were decreased in the TCDD exposed mice, suggesting a decreased bone formation. The osteoclast markers were normal, indicating normal osteoclast differentiation and resorptive activity. The impaired mineralization was explained by reduced osteoblast activity caused by increased level of the active form of vitamin D₃ [94]. Since osteoclast differentiation markers and osteoclast numbers were normal, TCDD might have more pronounced effects on the osteoblasts in males.

PCB 126 is one of the most potent dioxin-like PCBs and a study performed with this PCB indicates that the endogenous estrogen level is important. PCB 126 and estrogen supplementation in ovariectomized rats resulted in increased TBV, while PCB 126 and

estrogen decreased the TBV in sham operated animals. The sham operated rats also displayed an increased cortical thickness when treated with PCB-126 alone [95]. Thus, dioxins might induce different effects in pre-pubertal compared to adult females.

Also other AhR-ligands have shown to interfere with bone tissue homeostasis. Pregnant mice were injected with a total dose of 3 mg 3-MC/kg bw and the fetuses were examined with whole-mount skeletal and histological examinations. Fetuses to 3-MC exposed dams displayed a delayed ossification in the cervical and thoracic vertebrae, while the more proximal bones, such as femur and tibia, displayed normal ossification [96]. However, it is important to note that 3-MC not is classified as a dioxin-like compound and is not only an AhR-ligand. 3-MC also directly binds and activates estrogen receptor α (ER α) [97].

In vitro

Both osteoblasts and osteoclasts have been shown to express the AhR *in vivo*, suggesting that both cell types are responsive to dioxins [98, 99]. 10 nM TCDD inhibited the post-confluent formation of bone nodules in primary osteoblasts from rats [100]. In addition, the post-proliferative activity of ALP and level of osteocalcin was inhibited by 10 nM TCDD. Inhibited osteoblast differentiation was also reported after exposure to 1 nM TCDD in the chick periosteal osteogenesis (CPO) model, where the activity of ALP was reduced by 80% and the mRNA expression by 50% [101]. The mRNA expression of BSP, OPN and collagen type I was decreased, as well as the protein level of collagen type I. Furthermore, the mineralization was dose-dependently reduced in the stromal bone marrow cell (SBMC) model exposed to TCDD [101]. The expression of AhR in differentiating osteoblasts from male mice peaked after ALP and before the induction of osteocalcin, indicating a role of AhR in normal osteoblast differentiation [99]. IGFBP-6 is a potent mitogen of osteoblasts, which was induced by TCDD in female rat fetal calvaria and in the human osteosarcoma cell line SaOS-2. The authors suggested that the growth inhibitory effects of TCDD on osteogenesis were due to the altered IGFBP-6 [102]. Also the expression and secretion of collagenase-3 in UMR-106 rat osteosarcoma cells were stimulated by TCDD. The same response was also elicited by estrogen, suggesting an estrogenic response of TCDD on osteoblasts [103]. 3-MC also inhibited osteoblast differentiation *in vitro* [96].

Only two *in vitro* studies have been performed with osteoclasts exposed to TCDD. The activity of primary mature rat osteoclasts was not affected in the presence of TCDD [98]. However, differentiation-cultures from male mice displayed an inhibited osteoclast formation when exposed to 100 fM TCDD [104]. Also, other AhR-ligands, such as 3-MC, have been shown to inhibit osteoclastogenesis [105-107].

In summary, bone tissue homeostasis *in vivo* is disturbed by dioxins and this is most likely to be mediated by effects on both osteoblasts and osteoclasts. The differentiation of osteoblasts and osteoclasts seem to be inhibited by TCDD. However, the activity of mature osteoclasts seems to be unaffected. Whether TCDD can influence mature osteoblasts or not remains unknown. Also the mechanisms behind the osteoblast and osteoclast toxicity are largely unknown.

Gender differences in bone tissue due to AhR ligands

The study performed with female and male offspring to female rats exposed to 1.0 µg TCDD/kg bw during gestation, postnatally and lactation concluded that no gender related differences was detected with regard to geometry, densitometry or mechanical properties of the bones [92]. In this study the rats were sacrificed at postnatal day 40 and therefore the investigated rats were not completely sexually mature. Thus, dioxin exposure does not seem to cause gender related bone alterations in sexually immature rats.

PRESENT INVESTIGATION

AIM OF THE THESIS

Dioxins are ubiquitously spread environmental pollutants known to cause reproductive and developmental defects as well as cancer. During the last decades it has become evident that also bone tissue is a target for dioxin exposure. Most effects of dioxins are mediated by the AhR, although non-genomic effects have also been observed.

Bone tissue homeostasis is dependent on numerous factors including sex hormones and estrogen, in particular, plays an important role. Dioxins display endocrine disrupting properties and especially interference of AhR with estrogen signaling pathways has been indicated. As a consequence, dioxins or a constitutively active AhR (CA-AhR) might influence bone tissue differently in males and females. Moreover, the mechanisms for toxicity of dioxins are largely unknown.

Thus, the overall aim of this thesis was to investigate potential gender related effects in bone tissue as a result of dioxin toxicity and further elucidate possible mechanisms involved. The specific aims for each project are listed below:

- The aim of paper I was to investigate the expression and activity of AhR in the osteosarcoma cell line UMR-106 after dioxin exposure. A further aim was to study the expression of the AhR-responsive and bone-related gene osteopontin as possibly involved in effects on bone tissue by dioxins.
- The aim of paper II was to investigate if and how acute exposure to dioxin affects bone tissue in a short-term perspective.
- The aim of paper III was to characterize the bone phenotype of the CA-AhR transgenic mice and identify possible differences between male and female mice.
- Based on the conclusions regarding AhR-regulated bone resorption in paper III, the aim of paper IV was to investigate potential effects of an activated AhR during osteoclast differentiation.

METHODOLOGICAL CONSIDERATIONS

Cell lines (Paper I and IV)

In paper I and IV two different cell lines were used. In paper I an osteosarcoma cell line, UMR-106 (ATCC number: CRL-1661™), from a Sprague-Dawley rat was used to study the effects of TCDD on an osteoblast-like cell. The cell line is a clonal derivative from the osteosarcoma induced by injection of radiophosphorous. UMR-106 cells express receptors for parathyroid hormone (PTH) and 1,25 vitamin D₃. The cells also display many features of the normal osteoblasts and can mineralize when cultured in osteogenic medium. UMR-106 cells also express ALP and osteocalcin [109].

In paper IV a monocyte/macrophage cell line, RAW 264.7 (ATCC number: TIB-71™), from an adult male BALB/c mouse was used to study the effects of TCDD during osteoclast differentiation. The cells come from a tumor induced by Abelson murine leukemia virus. RAW 264.7 express RANKL and form multinucleated TRAP-positive osteoclast-like cells when treated with RANKL [110]. The differentiated cells also express the osteoclast markers calcitonin receptor (CTR), cathepsin K and MMP-9 [111].

Although these cell lines display many features of osteoblasts and osteoclasts it is important to remember that cell lines are immortalized and originate from a cancerous tissue. However, they are powerful tools when investigating molecular mechanisms and for screening purposes.

CA-AhR mouse model (Paper III-IV)

The construct encoding the mutant receptor was subcloned into a vector containing a strong promoter (SR α), which is a modified viral SV40 promoter and the E μ enhancer of the immunoglobulin heavy chain [112]. This construct also contains a poly adenylation site (pA) as well as a splicing region from the SV40 large T gene to increase the expression (Figure 3 on page 7). The transgenic mice were created by pronuclear injection of C57BL/6 x CBA eggs and then backcrossed into C57BL/6 resulting in a congenic C57BL/6 background. Therefore C57BL/6N was used as control (wild type) animals. The transgenic receptor is expressed in every organ examined so far [39, 40]. The transgenic mice also express the endogenous receptor, however, this is not activated since no ligand has been administered to the animals.

The activity of the CA-AhR, estimated from hepatic CYP1A1 induction, corresponds to the CYP1A1 induction in wild type mice exposed to a single dose of 0.3 µg TCDD/kg bw, which reflects a rather low activity of the receptor. Furthermore, the CA-AhR is expressed early in embryo development and throughout life.

Molecular analysis of the human AhR showed that the human AhR shares key mutations with the mouse DBA-type AhR and the DBA strain has therefore been suggested to be a more suitable model for studying the effects of dioxins [113]. However, the genetic background in the CA-AhR mice is C57BL/6, which is a dioxin sensitive mouse strain. The C57BL/6 encodes the alanine in codon 375 in the ligand binding domain, resulting in a 10 fold higher ligand binding affinity for TCDD, while the DBA-type carries valine in this position. However, the binding affinity of human AhR for TCDD varies at least 10 fold within the human population [114]. Thus, a genetic background of C57BL/6 might be more appropriate when extrapolating to humans, especially when considering risk groups and extra sensitive individuals. Taken together, these characteristics of the transgenic mouse model make it a relevant model for the human exposure situation.

Peripheral quantitative computed tomography – pQCT (Paper II-III)

Peripheral quantitative computed tomography (pQCT) is a three dimensional X-ray that records geometrical and dimensional properties of the intact bone. It is a well established technique that can perform separate estimates cortical and trabecular bone tissue and measures the true volumetric mineral density [115]. Another frequently used method for measuring bone mineral density is dual-energy X-ray (DEXA), however DEXA only measures the bone in two dimensions and can therefore only extrapolate the volumetric mineral density. pQCT measurement gives a lot of information of which parameters that might be altered by a specific treatment, however it cannot determine how or if the physical strength of the bones also have changed. Thus, an appropriate complement to this method is the three point bending test.

Three point bending test (Paper II-III)

After the pQCT measurements, the strength of the bones was tested with three point bending test. This is the most common method for testing the strength of long bones. The strength of the bones is dependent on many factors including mineral content, geometrical dimensions of the bones as well as the microarchitecture. The bone was

placed on two supports and a load was applied on the mid-diaphyseal area of the bone until failure. The three-point bending fixture was connected to a computer that registered load (N) and displacement (mm). Based on the load and displacement data it is possible to determine load at failure (N), displacement at failure (mm), energy to failure (J) and stiffness (N/mm) from the load-deflection curve. The three-point bending test reflects the actual strength of the bones, but it cannot determine exactly which parameters within the bone that have changed.

Quantitative morphology – stereology (Paper III)

Stereology is based on the principles of histomorphometry and quantifies three-dimensional structures from measurements of the structure on two-dimensional sections. The method can be used to calculate areas, volumes as well as the numbers and areas of specific cell types on sections from the tissue. The quantity of the structure is estimated in relative terms, i.e., densities. The density is defined as the quantity per volume, area or length within a defined space (tissue volume).

We used a transgenic mouse model to investigate the effects of a constitutively active AhR on trabecular bone tissue. The volume densities of trabecular bone and osteoclasts were quantified by point counting the micrographs of bone tissue sections using a square lattice. Trabecular bone density was calculated as the ratio of the points falling on trabecular bone to all the points falling on the tissue volume.

Serum analyses (Paper II-III)

Bone turnover can be estimated by looking at bone formation markers and bone resorption markers in serum. However, a drawback with serum analyses is that the level reflects the entire system in an organism. Thus, in most cases it is impossible to determine the specific response in a certain organ. However, the C-telopeptide cross-link of type 1 collagen (CTX) is a specific marker for bone resorption that can be measured in serum. CTX is generated by cleavage of the collagen molecule by cathepsin K, which is an event specifically performed by the active osteoclasts in bone. Thus, CTX measures the resorptive activity of the osteoclasts. While CTX measures the activity of the osteoclasts, the serum level of TRAP 5b has in several studies been shown to reflect the number of osteoclasts. [116-119]. The ratio of CTX and TRAP 5b thereby reflects the resorptive activity normalized to the numbers of osteoclasts and may subsequently indicate the individual resorptive activity of each osteoclast.

The serum level of the N-terminal propeptide of type I procollagen (PINP) is suggested to reflect the bone formation. However, collagen type I is ubiquitously expressed and PINP might reflect systemic disturbances in the production of collagen type I.

qPCR (Paper I, III and IV)

Gene expression analysis was performed with quantitative PCR (qPCR), which is a very sensitive method that detects small variations in mRNA levels of target genes in comparison to reference genes. The sensitivity of the method greatly depends on the selection of an appropriate reference gene. It is important that the treatment does not affect the gene expression of the reference gene. However, a limitation with this method is that the mRNA level of a target gene does not automatically correspond to the protein level. Thus is important to analyze especially important genes with other additional methods to confirm the results on a functional level, e.g., with Western Blot or protein activity assays.

RESULTS AND DISCUSSION

Decreased expression of osteopontin in a rat cell line exposed to dioxin Paper I

Studies with osteoblasts or osteoblast-like cells have suggested that dioxins and other AhR-ligands interfere with osteoblast differentiation [96, 99-102, 104, 120], however the mechanisms are not fully understood. In Paper I the osteoblast-like cell line UMR-106 was used to explore potential mechanisms of the toxicity of dioxins on osteoblasts.

Expression of the AhR at the mRNA level had previously been detected in the pre-osteoblast cell line MCT3T-E1 and in rat calvarial osteoblast-like cells (ROB cells) [96]. UMR-106 was found to express the AhR (Figure 1, Paper I), suggesting that UMR-106 cells are potentially responsive to TCDD. Furthermore, the marker gene for AhR activity, CYP1A1, was induced (Figure 2, Paper I), confirming that the AhR is activated in UMR-106 cells exposed to TCDD. Thus, TCDD is likely to evoke altered expression of dioxin-responsive genes in osteoblast-like UMR-106 cells. It was later shown that rat osteoblasts express AhR *in vivo* [99].

In bone tissue, the non-collagenous protein osteopontin (OPN) is produced by the osteoblasts in stages just prior to mineralization. OPN binds to the receptor $\alpha_v\beta_3$, which is abundantly expressed on the osteoclasts. The exact role(s) is not yet fully understood, but OPN has been suggested to be involved in several processes important for normal bone tissue homeostasis, e.g. mineralization, proliferation, migration of bone cells and bone resorption [121]. Interestingly, OPN deficiency protected from bone loss observed in aging female mice, while no difference was observed in the male mice [122]. Moreover, OPN deficient mice are resistant to bone loss caused by ovariectomy [123]. In addition, estrogen induces the expression of OPN, further implying hormonal influences in OPN function/regulation.

The mRNA expression, estimated by semi-quantitative RT-PCR, of the osteoblast differentiation markers, ALP, BSP II, collagen and OPN was decreased in chick periosteal osteogenesis (CPO) model exposed to 10 nM TCDD [101]. OPN was also identified as a target gene in transgenic mice with a constitutively active AhR, where the expression of OPN was reduced in stomach tumors of these mice [43]. UMR-106 cells also displayed decreased expression of OPN following TCDD exposure (Figure 3, Paper I). The decreased expression of OPN was more rapid and sensitive compared to

the induction of CYP1A1. The expression level of OPN was decreased after 6 hours at 1 pM TCDD, while CYP1A1 was induced after 12 hours at 100 pM TCDD. Thus, OPN is a more sensitive marker for TCDD exposure in UMR-106 cells than the classical TCDD marker gene CYP1A1.

A previous study with UMR-106 cells showed that the secretion of collagenase-3 displayed a similar response to estrogen and TCDD, suggesting that TCDD has estrogenic effects on collagenase-3 secretion [103]. Estrogen has been shown to induce the transcription of osteopontin in osteoblasts from mouse [124] and UMR-106 cells have been shown to express estrogen receptors [125]. Thus, the decreased expression of OPN in paper I suggests an anti-estrogenic effect of TCDD on OPN expression in UMR-106. The decreased expression of OPN might result in a decreased capacity of the osteoclasts to attach to the bone surface and thereby inhibit bone resorption or favor increased mineralization since OPN can act as an inhibitor of mineralization [126].

Acute exposure to TCDD *in vivo* affects bone tissue after only five days

Paper II

Acute toxic effects of TCDD include chloracne and wasting syndrome. However, bone tissue has not previously been studied with regard to short term exposure to dioxins. Male Sprague-Dawley rats exposed to a single high dose of TCDD displayed alterations in the trabecular part of the bone only five days after of exposure to TCDD. The total BMD in the metaphysis was increased, while the trabecular cross sectional area (CSA) was decreased (Table 1A, Paper II). No changes were detected in the cortical part of the bone (Table 1B and Table 2, Paper II), however changes at the cortical site was not expected after 5 days since the remodeling rate of cortical bone is slow.

Also serum markers for bone remodeling were altered in response to dioxin exposure. The bone formation marker PINP was decreased in TCDD exposed rats (Figure 1A, Paper II), suggesting that bone formation is decreased by TCDD. Decreased formation of bone might be due to decreased differentiation of osteoblasts, which would fit well with the *in vitro* studies showing that osteoblastogenesis is inhibited by dioxins [96, 99-102, 104, 120]. However, it is unlikely that effects on differentiation of osteoblasts would be detectable after only five days exposure *in vivo*, especially since the life-span of an osteoblast can be 3 month [127]. A more likely explanation would be increased

death of osteoblasts by apoptosis. It is also important to remember that PINP is a proteolytic fragment from the pro collagen type I molecule and that collagen type I is not specific to bone. On the contrary, collagen type I is ubiquitously expressed and a decreased PINP level might therefore reflect other systemic disturbances of collagen bio-synthesis and turn-over induced by an active AhR.

The bone resorption marker CTX was increased in TCDD exposed rats (Figure 1B, Paper II) indicating an elevated activity of the osteoclasts. This can be mediated by increased number of osteoclasts or enhanced resorptive activity per osteoclast. An *in vitro* study showed that the activity of mature osteoclasts was not disturbed by TCDD [98], indicating that the results in paper II can be explained by an increased differentiation of osteoclasts.

The mineral composition of the bones was altered in rats exposed to TCDD. The degree of mineralization was not changed but the relative amount of crystalline phosphate was increased, while the relative amount of acid phosphate was decreased in the TCDD treated rats (Table 3, Paper II). Thus, the mineral composition of bones from TCDD resembled the situation in more mature bone tissue. The mineralization process of calcified tissues (e.g. bone and teeth) has been suggested to be affected by TCDD in other *in vitro* and *in vivo* studies (refs), however the mechanisms remain unknown. One protein suggested to be involved in mineralization is the osteoblast-produced protein osteopontin. Purified bovine osteopontin has been shown to inhibit apatite formation and growth [128], bind to hydroxyapatite crystals and modulate bone crystal maturation [129, 130]. The expression of osteopontin was decreased as a result of TCDD exposure in UMR-106 cells (Paper I). The effects on mineralization in Paper II further support the notion that the osteoblasts are affected, either by an inhibited activity of the osteoblasts or increased osteoblast cell death.

In summary, the results in Paper II imply a direct or indirect impact on mature osteoblasts and an increased differentiation of osteoclasts by TCDD, which is observed after only five days exposure.

An activated AhR results in gender-specific bone responses

Papers III and IV

The importance of a functional AhR in dioxin mediated bone toxicity was demonstrated by studying rats with different AhR structures [90]. The rat strain with a dysfunctional transactivation domain (Han/Wistar) displayed resistance to dioxin exposure, while the bone tissue in rats with a functional AhR (Long-Evans) was considerably more affected. Bones from female Long-Evans rats displayed altered growth, modeling as well as physical properties when exposed to TCDD. The Han/Wistar strain displayed alterations in a few parameters, albeit at much higher doses of TCDD [90].

In paper III and IV a transgenic mouse model with a constitutively active AhR (CA-AhR) was used to investigate the long term effects of an activated AhR in bone tissue and during osteoclast differentiation. In paper III the bone phenotype of female and male transgenic mice with a constitutively active AhR (CA-AhR) was investigated, where the females were found to be more strongly affected than the males. Based on the findings in paper III, an *in vitro* study of osteoclast differentiation was conducted on bone marrow cells from CA-AhR females and males (Paper IV). In Paper IV osteoclast differentiation was also assessed in the RAW 264.7 cell line and in bone marrow cells from female and male wild type mice exposed to TCDD. The TCDD exposure can be viewed as an acute exposure in comparison to the chronic exposure in the CA-AhR mice.

Females

The CA-AhR females displayed changes both at trabecular and cortical parts of tibia. The trabecular area and trabecular bone mineral content (BMC) were increased in CA-AhR females, while the trabecular BMD was unchanged (Table 2, paper III). However, an increased trabecular area and content might be recorded even though there is an increased resorption. This is because endocortical resorption can lower the volumetric cortical BMD at the inner surface so it falls below the threshold value for cortical bone and falls into the range for trabecular bone [115].

The cortical part of the bone displayed slightly wider bones with a decreased density (Table 3, paper III). Thus, at the cortical site there seems to be an elevated bone resorption, a decreased bone formation or a combination of both. In contrast to the

results in paper III, female rats exposed to 1.7 μg TCDD/kg bw displayed smaller bones, both with regard to the length and the width of the tibia compared to the control animals [90]. The conflicting results might be due to species differences, type of exposure or stage of sexual maturation when starting exposure. The transgenic mouse model mimics a continuous low dose exposure present during embryo development, while the TCDD exposure is more acute and leaving less possibility for adaptive responses. The effects in the transgenic mice are the long-term effects, while the effects in the TCDD exposed animals reflect early responses. Also the activity of the AhR is likely to be different. The activity of the CA-AhR has been estimated to mimic a single dose of 0.3 μg TCDD/kg bw, which is almost six fold lower than 1.7 μg TCDD/kg bw. Moreover, the CA-AhR has been active since early embryo development, while the TCDD exposure started when the rats were 10 weeks and lasted for 20 weeks [90]. Another aspect could be that TCDD has been shown to elicit AhR independent non-genomic responses [53], which not would be possible in the transgenic mouse. Female offspring exposed *in utero* to a single maternal dose of 1.0 μg TCDD/kg bw on gestational day 11 or 15 displayed a reduced cortical BMD [92], which is in agreement with the findings in Paper III.

Alterations in the cortical part of bones from female CA-AhR mice were also reflected in the physical properties of the bones, where the results suggest softer bones (Table 4, Paper III). The stiffness of the bones from female rats exposed to TCDD was also reduced at a concentration of 17 μg TCDD/kg bw [90]. Thus, the stiffness of the bones seems to be affected similarly by a constitutively active AhR and by an acute TCDD exposure in female mice. Altered physical properties of the bones can be due to altered content, altered dimensions or altered quality of the bone tissue. All three of these factors likely contribute to the phenotype observed in female CA-AhR in Paper III. The volumetric cortical BMD (content) decreased, the bones became slightly wider (dimensions) and softer (quality). Hence, a constitutively active AhR interferes with female bone tissue homeostasis in multiple ways.

Analysis of the serum markers CTX and TRAP 5b suggest that the mean osteoclast activity was increased in the female CA-AhR mice (Figure 1, Paper III). Thus, the increased resorption in the transgenic females is likely to be mediated by an elevated osteoclast activity rather than increased number of osteoclasts. Furthermore, the osteoclast volume density was increased in the female transgenic mice (Figure 5B,

Paper III). Osteoclast size is positively related to osteoclast activity, i.e. larger osteoclasts resorb more actively [131-133]. Hence, in the transgenic female mice the bone resorption seems to be elevated, resulting in a decreased bone mass. Interestingly, the mRNA expression levels of several osteoclast genes involved in the function of osteoclasts (e.g. TRAP, cathepsin K and MMP-9) were decreased in female CA-AhR (Table 5, Paper III). The expression levels of the early osteoclast differentiation marker were, however, normal. Moreover, the RANKL/OPG ratio was increased indicating a slightly elevated drive for osteoclast formation. It is possible that increased osteoclast differentiation and elevated bone resorption can activate regulatory feed-back mechanisms to decrease the activity of the mature osteoclasts in an attempt to normalize the resorption.

In vitro osteoclast differentiation in bone marrow cultures from CA-AhR females displayed an increased number of osteoclasts (Figure 6A, paper IV). Also the mRNA expression of cathepsin K, MMP-9 and CTR genes were increased in the transgenic females (Table 4, paper IV). Thus a CA-AhR in female mice seems to increase the differentiation of osteoclasts, which also was indicated by the RANKL/OPG ratio in Paper III. However, it needs to be established, by differentiation of bone marrow cells from female CA-AhR to osteoclasts on bone slices, if the resorptive activity of these osteoclasts is increased or not.

A somewhat different pattern was observed in osteoclast differentiation cultures from female wild type mice exposed to TCDD, where the number of osteoclasts was unchanged (Figure 3A, Paper IV). However, the activity and protein level of TRAP as well as the gene expression of cathepsin K, MMP-9 and CTR were decreased in the presence of 10 and 1000 pM TCDD, respectively (Figure 4A, B and Table 2, Paper IV), indicating decreased osteoclast activity. Thus, *in vitro* differentiation of female osteoclasts with a CA-AhR seems to lead to opposite outcomes compared to osteoclast differentiation cultures from wild type females exposed to TCDD. Initially the differentiation of osteoclasts might be inhibited by acute exposure by TCDD in females, but when the exposure becomes chronic osteoclast differentiation is increased.

The serum level of the bone formation marker PINP was increased, while ALP, another bone formation marker, was decreased in bone extracts from female mice (Figure 2, Paper III). The mRNA expression levels of collagen type I and other osteoblast

markers, e.g. Runx2, was decreased in bones from the female CA-AhR mice (Table 5, Paper III), suggesting AhR mediated disturbances on bone formation. Inhibited osteoblast differentiation by dioxins has previously been demonstrated [96, 99-102, 104, 120], although the mechanisms still remain unknown. Runx2 is the master gene for osteoblast differentiation and this factor displayed a decreased mRNA expression in CA-AhR females. Hence, it is possible that Runx2 is one of the key mediators in dioxin-induced inhibition of osteoblast differentiation.

Males

The bone phenotype of male transgenic mice with a constitutively active AhR displayed almost no differences when compared to the wild type males. In total two parameters were altered; the trabecular BMD and the TRAP activity (Table 2 and Figure 4B, Paper III). However, *in vitro* differentiation of osteoclasts from male mice was remarkably affected by a CA-AhR and TCDD.

Male transgenic mice with a constitutively active AhR displayed an increased trabecular BMD, however, this did not alter the physical properties of the bones (Table 2 and 4, Paper III). A similar response was seen in male rats after five days exposure to a single high dose of TCDD (Paper II). Male mice exposed to TCDD via lactation (maternal dose of 15 µg TCDD/kg bw), displayed a dramatic increase in unmineralized osteoid and a reduced amount of mineralized bone in the metaphysis, suggesting a disturbed mineralization process [94]. Thus, acute exposure to TCDD and a continuously active AhR seem to induce a similar response in the metaphysis of male mice, with increased trabecular bone formation. The transgenic male CA-AhR mice did not display any alterations in the diaphysis, however male rats exposed to TCDD *in utero* (maternal dose of 1 µg TCDD/kg bw) displayed decreased cross sectional area and cortical BMD in the diaphysis of tibia if they were exposed between gestation days 11-19 [92].

The second parameter that was changed in male CA-AhR mice was the TRAP activity, which was decreased in bone extracts (Figure 4B, paper III). A decreased TRAP activity was also observed in the *in vitro* differentiation of osteoclasts from male CA-AhR (Figure 8C, Paper IV), where the gene expression of TRAP, RANK, cathepsin K, MMP-9 and CTR also was decreased. In contrast to the *in vitro* observations in Paper IV, osteoclast volume density was not different in male CA-AhR mice compared to

wild type male mice (Figure 5B, Paper IV). A similar effect was also observed in male mice exposed to TCDD through lactation, where the RANKL/OPG ratio and osteoclast numbers were normal. This collectively suggests that the inhibitory effect of a CA-AhR *in vitro* is somehow counteracted by yet unknown factors *in vivo*.

Similar to the *in vitro* osteoclast differentiation cultures from male CA-AhR mice, TCDD exposure of wild type male osteoclast differentiation cultures resulted in an inhibited osteoclast formation (Figure 4A, Paper IV). Also the expression levels of several osteoclast markers, both for differentiation and function, were decreased (Table 3, Paper IV). A short-term study of osteoclast activity showed that the activity of mature osteoclasts was not altered by TCDD [98]. However a recent study showed that 100 fM TCDD was sufficient to inhibit the differentiation of osteoclasts from male mice [104]. *In vitro* differentiation of osteoclasts from male mice was also inhibited by 3-MC [105]. Both a constitutively active AhR and acute TCDD exposure of male osteoclast differentiation cultures *in vitro* seem to cause an inhibited osteoclast differentiation.

Females versus males

A constitutively activated AhR from early development throughout life results in an increased bone resorption, possibly mediated by increased osteoclast differentiation and osteoclast activity, in female mice, but not in males. Gender-related differences in bone tissue have recently been described in Rhesus monkeys exposed to TCDD, where the females seem to be more affected than the male monkeys [93]. Also a study with pregnant sheep exposed to the non-dioxin-like PCB 153 displayed gender differences in the offspring, where the females exposed *in utero* seemed to be more severely affected than the males [108]. Interestingly, two epidemiological studies in Sweden have implied a similar response in humans in cohorts of Swedish fishermen and their wives from the Swedish east coast (exposed) and west coast (unexposed). The first study showed that women, but not the men, on the east coast had a significantly increased risk for vertebral fractures [134]. The second study with these cohorts showed that the east coast wives, with a higher intake of fatty fish, had an increased fracture incidence compared to the east coast wives consuming less fatty fish. No such association was detected in the men [135].

The results from Paper III and IV suggest gender-specific responses to a constitutively active AhR in bone tissue and in osteoclast differentiation *in vitro*. This could be mediated by cross talk between the AhR pathway and the estrogen signaling system, which is well documented [54, 63, 136-139]. One hypothesis is that dioxins act estrogen-like when the endogenous estrogen level is low, such as in males and anti-estrogenic when the endogenous estrogen level is high, such as in females. This hypothesis fits well with the results in Paper III and IV. Moreover, local production of estrogens in males might also be a part of the explanation. Aromatase (CYP 19) converts androgens into estrogens and peripheral aromatization of androgens is the major source of estrogen in males. In bone, aromatase is expressed in the osteoblasts but not in the osteoclasts [140]. Thus, aromatase activity in the osteoblasts of transgenic male mice could perhaps rescue the anti-estrogenic effects of the CA-AhR.

CONCLUSIONS

The overall conclusion of the present thesis is that bone tissue, as well as the individual cell types in bone tissue are sensitive targets for dioxin exposure and that females might be more responsive to an activation of the AhR compared to males.

In Paper I we showed that UMR-106 cells express the AhR and identified osteopontin as a more sensitive marker for dioxin exposure compared to the traditional dioxin marker gene CYP1A1. The decrease in osteopontin gene expression was observed earlier and at lower concentrations of TCDD than the induction of CYP1A1. A decreased osteopontin expression might alter the capacity of the osteoclasts to attach to the bone surface, but can also cause disturbances in mineralization.

In Paper II male rats exposed to a high single dose of TCDD displayed alterations in the trabecular bone tissue and in the mineral composition after only five days exposure to TCDD. Such a rapid response in bone tissue really high-lights the notion that bone is a highly dynamic tissue and also very responsive to dioxins.

In Paper III female mice with a constitutively active AhR (CA-AhR) were considerably more affected by an activated AhR, which resulted in bone loss correlated to an increased bone resorption. These results correlated to the observations *in vitro* (Paper IV), where osteoclastogenesis was elevated in cultures from transgenic females. The bone phenotype in male transgenic mice was almost unaffected, but osteoclast formation *in vitro* was inhibited in cultures from male CA-AhR mice. The different response *in vivo* and *in vitro* might be explained by yet unknown compensatory mechanisms present in male mice. Dioxins display endocrine disrupting properties and effects on the estrogen signaling system has been well established. In absence of estrogen, dioxins seem to have estrogenic properties, while in presence of estrogen, dioxins seem to display anti-estrogenic effects. Thus, the most obvious, and perhaps most likely reason for the gender specific responses is different endogenous estrogen levels in females and males. Increased bone resorption and bone loss is a well known feature of estrogen deficiency, thus the activated AhR in females in Paper III might cause anti-estrogenic effects.

Similar to the male CA-AhR mice, *in vitro* osteoclast formation in cultures from male wild type mice, was inhibited when exposed to TCDD. However, in contrast to the

elevated osteoclast differentiation in female CA-AhR, osteoclast differentiation in cell cultures from female wild type mice did not display an increased osteoclast formation during TCDD exposure. This might be explained by non-genomic effects of TCDD, such as activation of the MAPK pathway, in the TCDD exposed female cultures. Another plausible explanation might be the timing and duration of exposure. The CA-AhR has been active continuously since early embryo development until adulthood, while the TCDD exposure was applied for a shorter period of time in adult mice. This discrepancy in the females raises the question whether the effects that are observed after a temporary dioxin exposure at a specific age really reflects sustained long term effects of dioxin.

Taken together, the results in the present thesis suggest that bone tissue homeostasis and the differentiation and activity of bone cells are targets for dioxins. Moreover, the response to dioxins seems to be gender specific, therefore further research on dioxin induced toxicity on bone would likely benefit from separate studies on females and males.

FUTURE PERSPECTIVES

Dioxin-induced inhibition on osteoblast differentiation has been established, however the mechanisms still remain unknown. We showed that osteopontin (OPN) is an early marker for dioxin induced toxicity in osteoblastic cells, however whether this decreased expression results in a defective resorption by the osteoclasts remains to be verified. Interestingly, the volume and the number of osteoclasts were increased in OPN deficient mice, while the resorptive capacity was decreased [122].

The finding that male rats exposed to a single high dose of TCDD display alterations in bone tissue already after five days exposure, really challenges the common idea that bone is a rigid tissue. It would be interesting to know the minimum time required to induce alterations in bone tissue. Moreover, this study was performed in male rats and from study III and IV females seem to be more severely affected, thus it would be interesting to assess if the female rats also were more rapidly and/or more severely affected after acute exposure than the male rats.

The gender-related differences in Paper III and IV are really fascinating and raise a lot of questions. As mentioned before, one hypothesis is that dioxins exert estrogenic properties when endogenous estrogen levels are low and anti-estrogenic features when endogenous estrogen levels are high. Thus, it would be interesting to study whether this bone phenotype also is established in younger females, before sexual maturity when the endogenous levels of estrogen are lower than in adults. It would also be interesting to investigate if this bone phenotype is sustained in older females and if the males are affected at an older age. Another exiting experiment would be to study the effects of ovariectomy in the transgenic mice. If the hypothesis is correct, a CA-AhR in ovariectomized females would display estrogenic effects when compared to ovariectomized wild type mice.

To further analyze the different response in the female osteoclast differentiation cultures from CA-AhR mice and from wild type mice exposed to TCDD, an AhR inhibitor could be used. One potential mechanism is the non-genomic effects that can be induced by TCDD. Pretreatment of cultures from wild type mice with an AhR antagonist prior to TCDD exposure would reveal if dioxin elicit non-genomic alterations in osteoclasts. If non-genomic effects cause the different responses, the next step could be to use specific inhibitors for the different kinases of the MAPK pathway.

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