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Osteopontin: A rapid and sensitive response to dioxin exposure in the osteoblastic cell line UMR-106 ☆

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Abstract

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is an endocrine disrupting environmental pollutant that, among other effects, affects bone tissue. TCDD modulates the transcription of various genes, e.g., CYP1A1, and the present study is a part of a project aiming at developing an in vitro model system for identifying biomarkers specific for dioxin-induced effects in osteoblasts. Osteopontin (OPN) is an adhesion protein, suggested to be important in bone remodeling and our results indicate that TCDD down-regulates the transcription of OPN in the osteoblastic cell line, UMR-106. The present study shows that UMR-106 expresses the AhR and that the expression of CYP1A1 is induced after exposure to TCDD, while down-regulation of OPN is an even more rapid response and a sensitive biomarker to TCDD exposure in this osteoblastic cell line. In conclusion, this osteoblastic cell line may be used as an in vitro model-system for studying dioxin-induced effects on osteoblasts.

Keywords: Osteopontin; Osteoblast; Dioxin; AhR; CYP1A1

Dioxins are widespread endocrine disrupting environmental pollutants known to cause a wide range of toxic effects, the most sensitive effects being immunosuppression, developmental and reproductive effects, and cancer [1]. Few studies have focused on the effects of endocrine disrupting pollutants on bone tissue and even fewer have studied the effects of dioxins on bone tissue [2–10]. Rather recently the effects of TCDD on bone tissue were investigated in rats and the results show that TCDD interferes with bone growth, bone modelling, and mechanical strength of the bone in a dose-dependent manner [3]. The rat fetus and neonatal are even more sensitive than the adult to the effects on bone and TCDD results in adverse changes in three aspects of bone quality; bone geometry, bone mineral density, and bone mechanical properties [6]. Studies with primary rat osteoblasts have shown that TCDD inhibits the early stages of differentiation of the osteoblasts [2]. Thus, it is clear that dioxins affect the bone tissue, but the underlying mechanisms remain unknown.

TCDD is the high-affinity ligand for the aryl hydrocarbon receptor (AhR), which is a ligand-activated cytosolic receptor and a member of the basic-helix–loop–helix (bHLH) and Per-Arnt-Sim (PAS) family of transcription factors. This family of proteins is a group of regulatory factors that respond to various environmental cues. Upon ligand binding the AhR interacts with the structurally related partner protein Ah receptor nuclear translocator (Arnt) to mediate transcriptional activation of various genes, typically CYP1A1, which function in the oxidative metabolism of xenobiotics [11].

Osteopontin (OPN) is one of the predominant non-collagenous proteins in bone tissue and is produced both

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by the bone-forming cells and the bone-digesting cells, the osteoblasts and the osteoclasts, respectively. OPN is suggested to be involved in the remodeling of bone tissue where it promotes adhesion of the bone cells to the bone surface. OPN is post-translationally modified by phosphorylation and glycosylation at numerous sites in the protein and can act both as a structural molecule and as a cytokine [12,13].

Bone tissue is regulated by several hormones and one of the most important is estrogen, which induces bone formation by inhibiting the production of factors that induce the osteoclasts. In females, estrogen is primarily produced by the ovaries and ovariectomy of animals will result in a severely compromised estrogen production. Osteoporosis is a well-known effect of estrogen depletion. Interestingly, in 1999 it was observed that osteopontin-deficient mice were resistant to ovariectomy-induced bone resorption [14]. Thus, OPN seems to play an essential role in bone loss caused by estrogen depletion.

OPN expression is induced by estrogen in vivo, even though no classical estrogen receptor elements (EREs) have been found in the promoter of OPN. However, related sequences that can interact with estrogen receptor α (ERα) have been identified [15].

TCDD alters the estrogen signalling and mainly anti-estrogenic, but also estrogenic, properties have been observed in different studies [2,16,17]. Recent findings suggest that the dioxin-like PCB-126 might have estrogenic or anti-estrogenic properties depending on the estrogen status [4,5].

The aim of the present study was to investigate the effect of TCDD on the osteoblastic cell line UMR-106 by studying the expression of the bone associated protein osteopontin and the classical dioxin biomarker CYP1A1. Another ambition with this study was to identify a suitable in vitro model-system for studying the mechanisms behind the toxicity of dioxins and dioxin-like compound on osteoblasts.

Methods

Cell line. UMR-106 is a well-differentiated osteosarcoma cell line from Sprague–Dawley rats that originates from osteoblasts. The cell line was a generous gift from Karin Anestål at the Department of Cell and Molecular Biology, Karolinska Institutet. The cells exhibit several properties of mature osteoblasts [18]. The cells are adherent and were cultured according to the methods reported by Witasp et al. [19]. Dulbecco's modified Eagle's medium (Gibco-BRL, Middlothial, UK) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U penicillin/ml, and 100 μl streptomycin/ml (Gibco) at 37 °C and 5% CO_2 was used. Subculturing of the cells was done at 100% confluency every third day by trypsination with 0.25% trypsin–EDTA.

Cell exposure. Cells were plated in 6-well plates with a density of 10^6 cells/cm^2 and allowed to attach for 24 h. Cells were washed with PBS and TCDD-containing medium was added. TCDD dissolved in DMSO was purchased from Cambridge Isotope Laboratories (US). The different concentrations of TCDD used for the PCRs were: 1, 100, 1000, and 10,000 pM, and were performed in triplicate of each concentration. The cells were incubated for 6, 12, or 24 h and were washed with PBS prior to the RNA isolation. In the cell proliferation assay and the cell viability experiment 0.001, 1, and 1000 pM were used. The cells were incubated for 6 or 24 h and washed with PBS.

RNA isolation. Total RNA was isolated with TRIzol Reagent (Invitrogen, UK). The absorbance was measured spectrophotometrically (BioPhotometer, Eppendorf AG, Germany) at 260 nm and the concentration of RNA was calculated.

DNase I treatment and cDNA synthesis. The samples from the RNA isolation were DNase I-treated (Invitrogen, UK) prior to PCRs in order to avoid contamination of genomic DNA. One microgram of total RNA was used in the reaction. cDNA synthesis was performed with Superscript RT (200 U/μl; Invitrogen, UK) and the cDNA was later used for PCR.

Reverse transcriptase PCR. cDNA, PCR-buffer, dNTPs, $MgCl_2$, primers, H_2O, and *Taq* polymerase were mixed in PCR tubes to a final volume of 25 μl. Primers against CYP1A1 [20], AhR [21], and β-actin [22] were used in different runs. The PCR conditions during the run consisted of 10 min at 94.5 °C, followed by 34 cycles of 94.5 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and finished with 10 min at 72 °C. The samples were stored in 4 °C until separated on an agarose gel (1%). Positive and negative controls as well as a 1kbp plus DNA ladder (Invitrogen, UK) were always included on the gel. The gel was examined on a UV light table and photographed.

Real time PCR. Sybr-green real time PCR was used to quantify the expression of CYP1A1 and OPN. The expression of CYP1A1 and osteopontin was standardized against β-actin. Primers (CYP1A1 [23] and β-actin [22]) purchased from Invitrogen, UK, were mixed with SYBR Green PCR Master Mix (Applied Biosystems) and cDNA, and the samples were run in a Smart Cycler (Cepheid, US). Primers against OPN were designed with Primo (Chang Bioscience) and sequence specificity was confirmed by Basic Local Alignment Search Tool (BLAST), National Center for Biotechnology Information (NCBI). The PCR conditions for CYP1A1 were: 95 °C 10 min, 95 °C 15 s, 58 °C 1 min, and 72 °C for 1 min. The conditions for β-actin and OPN were: 95 °C 10 min, 95 °C 15 s, 55 °C 1 min, and 72 °C for 1 min. Triplicates of each concentration of TCDD were analyzed, except for the CYP1A1 mRNA quantification. The results from the 24 h exposure to TCDD are based on duplicate from the control, 100, and 10,000 pM.

Cell viability. The effect of TCDD on cell viability was investigated by staining the cells with trypan blue and counting the cells. Cells were plated in 6-well plates with a density of 20,000 cells/cm^2 and allowed to attach for 24 h. After 6 and 24 h exposure to TCDD (0.001, 1, and 1000 pM), the cells were washed with PBS (Gibco), followed by trypsination with 0.25% trypsin–EDTA. The cells were stained with 0.5% trypan blue and counted under a Bürker chamber in a light microscope. Triplicates of each concentration were analyzed and the percent of viable cells and dead cells was compared. The experiment was repeated four times.

Cell proliferation. Cell proliferation during exposure to TCDD was studied with a [^{3}H]thymidine incorporation assay. Cells were plated and treated like the cell viability experiment, but 6 h prior to harvesting 1.0 μl [^{3}H]thymidine solution (*methyl*-[^{3}H]thymidine, Amersham Bioscience UK, Buckinghamshire, UK) was added. The cells were washed twice with ice-cold PBS and twice with 0.6 ml TCA (5%). The cells were collected in 0.5 ml of 0.5 M NaOH and stored in −20 °C for later analysis. For analysis the samples were counted in a scintillation counter (Beckman Instruments, UK). Triplicates of each concentration of TCDD were analyzed and the experiment was repeated three times.

Statistics. Statistics were performed with Student's *t* test.

Results and discussion

Few studies have focused on how dioxins influence bone tissue [2–8]. In vivo and in vitro studies performed to date have shown that bone is negatively affected by dioxin and dioxin-like compounds, but the underlying mechanisms remain unknown. In this study, we demonstrate that OPN, which is an adhesion molecule that promotes attachment of bone cells to the bone surface, is affected by TCDD and our hypothesis is that OPN is involved in an altered bone turnover caused by dioxins.

UMR-106 is a highly differentiated osteoblastic cell line from rat and this study shows that TCDD neither alter the cell viability nor the cell proliferation during the first 24 h of exposure in the cells (data not shown). These results are supported by a study performed by Gierthy and co-workers in 1994 [2], which also demonstrated that cell proliferation is unaffected by TCDD in this specific cell line.

The toxic effects of dioxins and dioxin-like compounds are mediated through signalling of AhR. The classical target gene for an active AhR and one of the most sensitive responses to dioxin is transcription of CYP1A1. However, CYP1A1 induction may not directly be related to the toxicity of dioxins [24]. The present study is the first to demonstrate that the osteoblast-like cell line UMR-106 expresses the AhR (Fig. 1). AhR has previously been reported to be expressed in both primary cultures of rat osteoblast-like cells (ROB) and mouse calvarial clonal preosteoblastic (MC3T3-E1) cells [25]. The expression of AhR in osteoblasts, both in primary cell cultures and in cell lines, implies that osteoblasts are potential targets for dioxin and dioxin-like compounds. Furthermore, the bone-resorbing osteoclasts also express the AhR [26], suggesting that dioxin and dioxin-like compounds can influence both the bone building process as well as the bone resorption. Thus, an imbalance between bone formation and bone resorption may result in an increased risk for osteoporosis or osteopetrosis.

In the present study, the constitutive expression of CYP1A1 in UMR-106 was very low or non-existing (Fig. 1). However, after 24 h exposure to 1000 pM TCDD the expression of CYP1A1 mRNA was induced. Thus, this is the first study which shows that CYP1A1 is expressed in this cell line and can be induced by exposure to TCDD. Quantification of the expression of CYP1A1 with real-time PCR shows that CYP1A1 is induced, but only slightly (Fig. 2) when compared to other cell types such as hepatocytes, which show a much greater induction of CYP1A1 when exposed to TCDD. The highest induction of

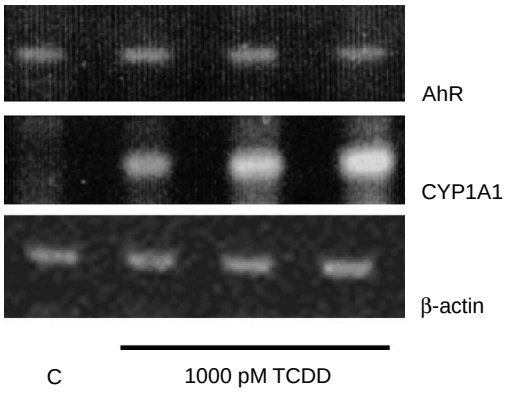

Fig. 1. RT-PCR shows that the osteoblastic cell line UMR-106 expresses the AhR and the expression of CYP1A1 is induced after 24 h exposure to 1000 pM TCDD. The first lane represents the control and lanes 2–4 correspond to exposure to 1000 pM TCDD. β-Actin was used as loading control.

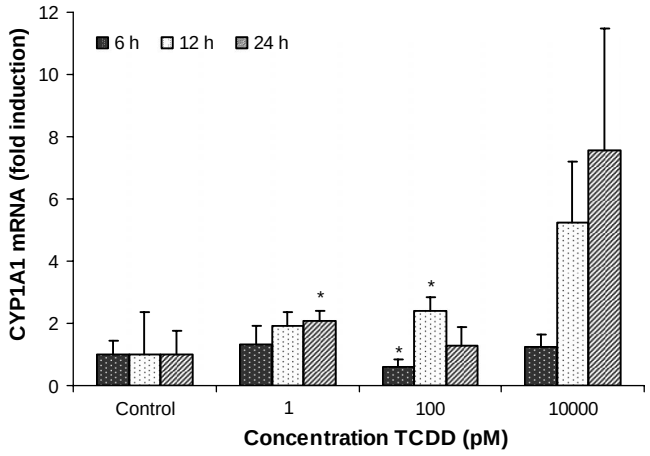

Fig. 2. Quantification with real time PCR shows that the expression of CYP1A1 mRNA is induced in the osteoblastic cell line UMR-106 after exposure to 1, 100, or 10,000 pM TCDD for 6, 12, or 24 h. (Analyzed from triplicates, but from 24 h exposure, the control, 100, and 10000 pM were analyzed from duplicates.) $*P < 0.05$.

CYP1A1 mRNA in the present study was roughly a 10-fold induction in the UMR-106 cells. However, it is intriguing to speculate that even a slight increase of CYP1A1 levels might have an in the micro-environment of osteoblasts. Remodeling of the bone tissue is performed by small contemporary units of active bone cells called basic multicellular units (BMUs). The BMU consists of osteoclasts in the front that digest the bone, and osteoblasts in the rear that secrete osteoid and thereby begin the synthesis of new bone tissue [27]. CYP1A1 metabolize estrogen and an increased amount of CYP1A1 enzyme (or any other dioxin-induced effect) in the most adjacent milieu of the BMUs might have an impact on the estrogen level closest to the BMUs. An increased metabolism of estrogen leads to less estrogen that can inhibit the osteoclasts, leading to a higher rate of bone resorption. Moreover, the osteoclasts express the AhR as well, suggesting that CYP1A1 potentially can be induced in the osteoclasts, leading to an even higher rate of estrogen metabolism in the near environment of the active bone cells. Bone loss and impaired bone tissue formation are well-known effects of estrogen deficiency.

One of the major non-collagenous proteins in bone tissue is OPN and our study shows that the expression of OPN was significantly decreased already after 6 and 12 h exposure to concentrations from 1 and 100 pM TCDD, respectively (Figs. 3A and B). No such effect was observed after 24 h exposure to TCDD (Fig. 3C). The response in osteopontin expression is much more rapid response to TCDD than the up-regulation of CYP1A1. Osteopontin seems to be a more rapid and sensitive marker for the early effects of TCDD in this osteoblastic cell line. In addition, OPN has been reported to be down-regulated in several organs (e.g., kidney, lung, and stomach) in transgenic mice with a constitutively active AhR, the CA-AhR mice. The CA-AhR mice mimic the response of wild type mice continuously exposed to low levels of dioxins [28].

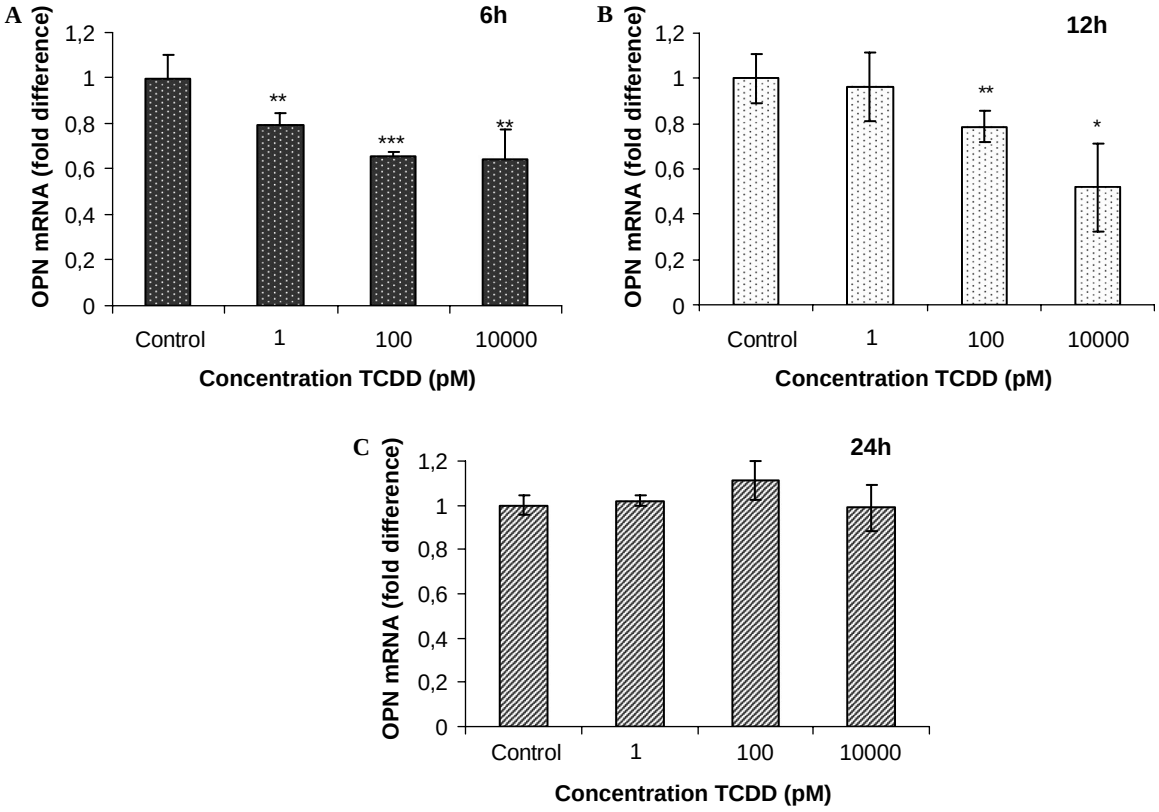

Fig. 3. Quantification with real time PCR shows that the expression of osteopontin mRNA in UMR-106 was significantly and dose-relatedly down-regulated after 6 h exposure to 1 pM TCDD (A) and 12 h exposure to 100 pM TCDD (B). However, the expression of osteopontin after 24 h appeared to be unaffected (C). $*P < 0.05$, $**P < 0.01$,$***P < 0.001$.

Estrogen has been shown to induce the transcription of OPN and elements that can interact with ERα have been reported [12,15]. In this study, it seems like TCDD has anti-estrogenic effects in the regulation of OPN in the UMR-106 cell line, since OPN is down-regulated. Furthermore, two putative dioxin responsive elements (DREs) have been identified in the mouse OPN gene. Four DRE motifs have also been found at similar positions in the human OPN gene [28], suggesting that the OPN gene can be directly regulated by TCDD. Thus, the expression of osteopontin might be regulated both by estrogen and TCDD directly. The decrease in OPN expression might be explained by direct regulation of the OPN gene by AhR, by cross-talk between the AhR signaling pathway and the ER signaling pathway, or both [29,30].

In conclusion, the present study shows that the osteoblast-like cell line UMR-106 expresses the AhR and that the expression of CYP1A1 is induced after exposure to TCDD, while OPN is down regulated. The expression of osteopontin is a more rapid response to TCDD exposure than CYP1A1 in UMR-106. So far, our findings indicate that this cell line can be used as a model-system for studying the effects of dioxin and dioxin-like compounds on osteoblasts, but further research and conformation of the findings in vivo are necessary.

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