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Aryl hydrocarbon receptor catabolic activity in bone metabolism is osteoclast dependent *in vivo*

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ABSTRACT

Bone mass is regulated by various molecules including endogenous factors as well as exogenous factors, such as nutrients and pollutants. Aryl hydrocarbon receptor (AhR) is known as a dioxin receptor and is responsible for various pathological and physiological processes. However, the role of AhR in bone homeostasis remains elusive because the cell type specific direct function of AhR has never been explored *in vivo*. Here, we show the cell type specific function of AhR *in vivo* in bone homeostasis. Systemic AhR knockout (AhRKO) mice exhibit increased bone mass with decreased resorption and decreased formation. Meanwhile, osteoclast specific AhRKO ($AhR^{\Delta Oc/\Delta Oc}$) mice have increased bone mass with reduced bone resorption, although the mice lacking AhR in osteoblasts have a normal bone phenotype. Even under pathological conditions, $AhR^{\Delta Oc/\Delta Oc}$ mice are resistant to sex hormone deficiency-induced bone loss resulting from increased bone resorption. Furthermore, 3-methylcholanthrene, an AhR agonist, induces low bone mass with increased bone resorption in control mice, but not in $AhR^{\Delta Oc/\Delta Oc}$ mice. Taken together, cell type specific *in vivo* evidence for AhR functions indicates that osteoclastic AhR plays a significant role in maintenance of bone homeostasis, suggesting that inhibition of AhR in osteoclasts can be beneficial in the treatment of osteoporosis.

1. Introduction

Bone mass is regulated by various endocrine factors, such as sex steroid hormones and several pituitary gland hormones, and by exogenous factors, such as nutrients and pollutants. Among these regulators, fat-soluble bioactive substances are recognized as significant molecules. It is well known that fat-soluble bioactive substances target to various tissues and organs, and play significant roles in the maintenance of physiological homeostasis. Fat-soluble bioactive substances are diverse, and include fat-soluble vitamins such as vitamin A/D, steroid hormones such as androgen and estrogen, and environmental toxins such as dioxins.

Fat-soluble bioactive substances act as ligands and bind to nuclear receptors. Nuclear receptors bind directly to specific DNA elements in target gene promoters and/or enhancers, and positively or negatively control transcription [1]. For example, vitamin D binds to the vitamin D receptor (VDR) and targets small intestine and kidney, regulates calcium metabolism, and participates in bone homeostasis [2,3]. Likewise, estrogen, a sex steroid hormone, regulates development and maturation of reproductive organs, and also maintains bone homeostasis directly or indirectly [4]. The physiological significance of nuclear receptors in bone metabolism has been revealed by studies focused on the function of cell type specific nuclear receptors [5,6].

Notably, fat-soluble bioactive substances such as dioxins also exert physiological actions by binding to the aryl hydrocarbon receptor (AhR). AhR, which is expressed ubiquitously, is a member of a transcription factor superfamily characterized by structural motifs of basic helix-loop-helix (bHLH)/Per-AhR nuclear translocator (Arnt)-Sim (PAS) domains [7]. The toxicology and pharmacology of AhR has been studied for over a decade. With recent discoveries of novel AhR functions, however, AhR research has expanded into multiple aspects of physiology and pathology, such as immunoregulation [8,9], tumor suppression [10], liver development [11], reproduction [12], and vascular remodeling of the developing embryo [13]. However, few studies have addressed the regulation of bone homeostasis by AhR. Hitherto, the function of AhR on bone

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metabolism has been reported by two research groups [14,15], however, these studies were analyzed using systemic AhR knockout or transgenic mice expressing constitutively active AhR. Systemic mutant mice might influenced by indirect effects from tissues/organs except bone, therefore, AhR function might be clarified by more focused studies utilizing conditional AhR knockout mice that can elucidate the bone cell type specific functions of AhR *in vivo*.

In this study, we analyzed mice in which the AhR gene had been conditionally deleted from osteoclasts to reveal that osteoclastic AhR plays a significant role in the maintenance of bone. Mice lacking AhR in osteoclasts ($AhR^{\Delta Oc/\Delta Oc}$) were resistant to bone loss induced by ovariectomy (OVX), orchidectomy (ORX), or treatment with 3MC, an AhR agonist, suggesting that control of AhR activity in the osteoclast might be a promising therapeutic strategy for osteoporosis.

2. Materials and Methods

2.1. Mice

Q2 Systemic AhR KO mice [16] and AhR floxed mice were provided by Dr. Y Fujii-Kuriyama. The $\alpha_1(I)$-*Collagen-Cre* mice ($Col1a1\text{-}Cre^{tg/0}$) were kindly provided by G. Karsenty Laboratory [17]. Osteoblast-specific AhR KO mice ($AhR^{\Delta Ob/\Delta Ob}$: $Col1a1\text{-}Cre^{tg/0}$; $AhR^{flox/flox}$) were generated by breeding $Col1a1\text{-}Cre^{tg/0}$; $AhR^{flox/+}$ male mice and $AhR^{flox/flox}$ female mice, and $AhR^{flox/flox}$ mice were considered as controls. The *Cathepsin K Cre* (*Ctsk-Cre*) mice ($Ctsk^{Cre/+}$) [5] were back-crossed into C57BL6 more than ten times. Osteoclast-specific AhR KO mice ($AhR^{\Delta Oc/\Delta Oc}$: $Ctsk^{Cre/+}$; $AhR^{flox/flox}$) were generated by breeding $Ctsk^{Cre/Cre}$; $AhR^{flox/+}$ male mice and $Ctsk^{+/+}$; $AhR^{flox/+}$ female mice, and $Ctsk^{Cre/+}$; $AhR^{+/+}$ were considered as controls. All mice were housed in a specific-pathogen-free (SPF) facility under climate-controlled conditions with a 12-h light/dark cycle and were provided with water and standard diet (CE-2, CLEA, Japan) *ad libitum*. They were euthanized at 12 or 24 weeks of age. All animals were maintained and examined according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo and Ehime University.

2.2. Micro-computed tomography (μCT) analysis

Micro-CT analysis was performed as described using a μCT system (μCT35, SCANCO Medical, Bruttisellen, Switzerland) [2,6]. Briefly, 466 slices were acquired, starting just beneath the end of the growth plate, thus including both the primary and secondary spongiosa, and a region 1.8 mm in length of the distal metaphyseal secondary spongiosa (300 slices) was selected for analysis. Three-dimensional reconstructions were generated and analyzed according to the guideline [18].

2.3. Bone histomorphometric analyses

Bone histomorphometry was performed as previously described [5,6]. Bone histomorphometric analyses were performed using the OsteoMeasure analysis system (OsteoMetrics Inc., GA, USA) according to ASBMR guidelines [19].

2.4. AhR-ligand treatment

Female control ($AhR^{+/+}$: $Ctsk^{Cre/+}$ $AhR^{+/+}$) and osteoclast-specific AhR KO ($AhR^{\Delta Oc/\Delta Oc}$: $Ctsk^{Cre/+}$; $AhR^{flox/flox}$) littermates were treated with 3-methylcholanthrene (3MC, Wako, Japan) or a placebo of corn oil starting when they were eight weeks old. 3MC was dissolved in corn oil and injected at 0.1 mg/g body weight, twice a week for four weeks. Mice were analyzed at 12 weeks of age.

2.5. Mice orchidectomy and ovariectomy model

Eight-week-old control mice ($AhR^{+/+}$: $Ctsk^{Cre/+}$ $AhR^{+/+}$) and osteoclast-specific AhR KO mice ($AhR^{\Delta Oc/\Delta Oc}$: $Ctsk^{Cre/+}$; $AhR^{flox/flox}$) were sham operated, orchidectomized, or ovariectomized under anesthesia. Four weeks after surgery, all of the mice were euthanized and subjected to micro-computed tomography and bone histomorphometry.

2.6. Elisa

ELISAs were performed following the manufacturers' protocols using the Estradiol EIA Kit (Cayman Chemical Company) for estradiol, Testosterone EIA Kit (Cayman Chemical Company) for testosterone, and Insulin-like Growth Factor-I (IGF-1) ELISA TEST (Endocrine Technologies) for IGF-1.

2.7. Osteoclast culture

Osteoclastogenesis *in vitro* was studied by plating bone-marrow cells from 8-week-old mice in culture dishes containing α-MEM (GIBCO) with 10% FBS (MP Biomedicals). After incubation for 8 h, nonadherent cells were collected, and cells were seeded (3×10^5 cells/dish) in 6 cm suspension dishes containing α-MEM with 10 ng/ml M-CSF (R&D Systems). After 2 days (about 80% confluent), adherent cells were used as osteoclast precursor cells after washing out the nonadherent cells. Cells were cultured in the presence of 10 ng/ml M-CSF and 234 ng/ml GST-RANKL (Oriental yeast, Japan) for three days to generate osteoclast like cells.

2.8. Real-time PCR analysis

Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using PrimeScript RT Master Mix (Takara Bio Inc) and subjected to real-time PCR using KAPA SYBR Fast qPCR Kits (Kapa Biosystems) with Thermal Cycler Dice (Takara Bio Inc) according to the manufacturers' instructions. Expression levels were normalized by *Gapdh*. The following primers were used: *AhR*, 5′-TTCTATGCTTCCTCCACTATCCA-3′ and 5′-GGCTTC GTCCACTCCTTGT-3′; *Gapdh*, 5′-AAATGGTGAAGGTCGGTGTG-3′ and 5′-TGAAGGGGTCGTTGATGG-3′.

2.9. Statistical analysis

Data were analyzed by a two-tailed student's *t*-test. For all graphs, data are represented as mean ± SD. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Increased bone mass in AhR KO ($AhR^{-/-}$) mice

We characterized the bone phenotype of mice in which AhR was systemically knocked out (AhRKO, $AhR^{-/-}$). We performed micro-computed tomography (μCT) on the distal femur to measure BMD and analyze three-dimensional trabecular architecture to determine whether there were differences between $AhR^{-/-}$ and $AhR^{+/+}$ mice. As shown in Fig. 1A–C, the distal femurs of both male and female $AhR^{-/-}$ mice exhibited greater trabecular bone volume (BV/TV), trabecular number (Tb.N), connectivity density (Conn.D), and bone mineral density (BMD), and smaller trabecular separation (Tb.Sp) and structure model index (SMI) when compared with $AhR^{+/+}$ mice. Next we performed Von Kossa/Van Gieson staining in the L3 and L4 lumbar vertebrae to assess mineralized bone volume in vertebral trabecular bone of $AhR^{-/-}$ mice. Consistent

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