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# Losartan increases bone mass and accelerates chondrocyte hypertrophy in developing skeleton

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ABSTRACT

Angiotensin receptor blockers (ARBs) are a group of anti-hypertensive drugs that are widely used to treat pediatric hypertension. Recent application of ARBs to treat diseases such as Marfan syndrome or Alport syndrome has shown positive outcomes in animal and human studies, suggesting a broader therapeutic potential for this class of drugs. Multiple studies have reported a benefit of ARBs on adult bone homeostasis; however, its effect on the growing skeleton in children is unknown. We investigated the effect of Losartan, an ARB, in regulating bone mass and cartilage during development in mice. Wild type mice were treated with Losartan from birth until 6 weeks of age, after which bones were collected for microCT and histomorphometric analyses. Losartan increased trabecular bone volume vs. tissue volume (a 98% increase) and cortical thickness (a 9% increase) in 6-weeks old wild type mice. The bone changes were attributed to decreased osteoclastogenesis as demonstrated by reduced osteoclast number per bone surface *in vivo* and suppressed osteoclast differentiation *in vitro*. At the molecular level, Angiotensin II-induced ERK1/2 phosphorylation in RAW cells was attenuated by Losartan. Similarly, RANKL-induced ERK1/2 phosphorylation was suppressed by Losartan, suggesting a convergence of RANKL and angiotensin signaling at the level of ERK1/2 regulation. To assess the effect of Losartan on cartilage development, we examined the cartilage phenotype of wild type mice treated with Losartan *in utero* from conception to 1 day of age. Growth plates of these mice showed an elongated hypertrophic chondrocyte zone and increased Col10a1 expression level, with minimal changes in chondrocyte proliferation. Altogether, inhibition of the angiotensin pathway by Losartan increases bone mass and accelerates chondrocyte hypertrophy in growth plate during skeletal development.



## 1. Introduction

Losartan, the first angiotensin receptor antagonist used to treat hypertension, has been used in pediatrics for blood pressure control. Human and animal studies have shown positive outcomes of this treatment on other disease models, for example in Marfan Syndrome, Angiotensin II blockage reduced aortic root dilation [1,2]; in Alport Syndrome for treatment of proteinuria [3]. However, the effect of Losartan on the growing skeleton is poorly understood. This is especially important as increasing numbers of children treated with this class of drugs are affected with conditions that predispose them to low bone mineral density (BMD), like in Marfan syndrome [4–6]. To better understand the role of Angiotensin signaling in the growing skeleton, we studied the effects of Losartan treatment during murine bone and cartilage development.

The renin–angiotensin pathway is a key regulator of cardiac physiology and electrolyte homeostasis. The peptide hormone angiotensin II (AngII) is produced through a series of regulated proteolytic processes. First synthesized in the liver, angiotensinogen is cleaved by renin in the kidney to release angiotensin I, an inactive decapeptide. Upon cleavage by angiotensin converting enzyme (ACE), angiotensin I is converted into the biologically active octapeptide — AngII [7]. AngII binds and stimulates type 1 (AGTR1) and type2 (AGTR2) angiotensin receptors, a class of the seven-transmembrane G-protein coupled proteins. In rodents, type 2 angiotensin receptor is formed by a single protein encoded

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by Agtr2 gene, while the type 1 angiotensin receptor is a receptor complex consisting of Agtr1a and Agtr1b receptors.

Multiple reports in adult mouse and rat models [8–10] have shown that blockage of Agtr1a or Agtr2 effectively increased bone mass under physiological conditions and in an ovariectomy (OVX) induced osteoporosis model. In contrast, one study showed that an ACE inhibitor reduced BMD in the distal femoral metaphysis in mice [11]. Agtr1a, Agtr1b, Agtr2 and ACE are expressed in osteoclasts and osteoblasts, the two key cell types controlling the balance between bone resorption and formation. Agtr1 inhibition *in vivo* attenuated the differentiation of monocytes, the precursor cells of osteoclasts [12]. In a rat cell line, blocking Agtr1 *in vitro* reduced osteoclastogenesis indirectly by increasing the ratio of RANKL/OPG in osteoblasts [10]. Collectively, these data support that angiotensin signaling influences bone remodeling in the adult skeleton.

Angiotensin converting enzyme inhibitor has been reported to inhibit the conversion of type II procollagen to collagen in cartilage culture [13]. The expression of AGTR1 and AGTR2 is found in human articular chondrocytes as well as articular chondrocytes from patients with osteoarthritis or rheumatoid arthritis. The expression of these receptors is up-regulated in response to IL-1, a key mediator in chronic and destructive arthritis and cartilage erosion [14], suggesting a role for AngII signaling in chondrocyte physiology as well as in pathogenic processes. However, there is no study that has demonstrated the function of these receptors on chondrocytes in the growth plate yet in growing skeleton.

To better understand the role of angiotensin signaling in bone and cartilage during development, we examined the bone and cartilage phenotypes of growing mice treated with Losartan. We show that Losartan can increase bone mass *in vivo* and directly suppress osteoclastogenesis *in vitro* accompanied by decreased RANKL mediated ERK phosphorylation in osteoclast. In the growth plate, Losartan leads to increased chondrocyte hypertrophy without changing resting chondrocyte proliferation *in vivo*.

## 2. Materials and methods

### 2.1. Animal studies

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Baylor College of Medicine (protocol # AN-1506) and were performed in strict accordance with the Guide for the Use and Care of Laboratory Animals of the National Institutes of Health.

### 2.2. Drug preparation and treatment with an AGTR1 receptor blocker

Losartan tablets (50 mg/tablet) were crushed with a mortar and pestle and were dissolved in water provided to mice at a dosage concentration of 0.6 g/L [2,15]. For the bone study, one day after delivery, wild type C57BL/6 lactating mothers were given oral Losartan (0.6 g/L) or water (standard drinking water) until weaning. After weaning, oral losartan treatment (0.6 g/L) or water was continued for the pups that had been previously treated via their lactating mothers. Both control and experimental mice were sacrificed at 6 weeks and their bones were collected for the different arms of experiments. For the cartilage study, wild type C57BL/6 dams were given oral Losartan (0.6 g/L) or water starting from the first day of conception till the day after the pups were born. The pups from treated or untreated mother were sacrificed at P1 and limbs were collected for histology.

### 2.3. Micro Computed Tomography (microCT) analysis

MicroCT analysis was performed on the femurs of 6-week-old C57BL/6 male wild-type mice treated with Losartan or water. A microCT system (Scanco microCT40, SCANCO Medical, Swiss) was used for imaging the bones at medium resolution of 12 μm. 3D reconstruction and image analysis was performed on the trabecular bone of the distal femurs using the integrated software of the Scanco microCT40 system.

### 2.4. Histomorphometric analysis

The lumbar spines and the femurs from the same 6-week-old mice were embedded in polymethylmethacralate plastic resin and sectioned at a 7-micrometer thickness. Coronal histological sections were then stained with tartrate resistant acid phosphatase (TRAP) to calculate osteoclastic parameters, with toludine blue to conduct osteoblast indices, and Von Kossa staining to perform bone volume to tissue volume (BV/TV) measurements. Osteometrix software was used to analyze these variables. For the measurement, one section was chosen from each lumber and femoral sample to make the location of the specimen consistent, and two observers were double-blinded to read the slides [16].

### 2.5. Immunohistochemistry analysis

Col10a1 immunofluorescence was performed with polyclonal rabbit antibody againstCol10a1 (pXNC2, a kind gift from Dr. Greg Lunstrum) with dilution of 1:1000 followed by secondary antibody Donkey-anti-Goat-Alexa (1:600). Agtr1 antibody for immunofluorescence was purchased from Santa Cruz (sc-1173) with dilution of 1:200 followed by secondary antibody Goat-anti-Rabbit-Alexa (1:600). The immunostaining protocol for Col10a1 and Agtr1 is the same as the standard protocol. BrdU incorporation assay and growth plate measurements were conducted as described before on the P1 limbs [17,18].

### 2.6. Osteoclast cell culture, TRAP staining and western Blot

Bone marrow cells from 4 to 6 week old mice were harvested. Adhesion cells were discarded and floating cells were cultured with M-CSF (#216-MC-005, R&D Systems) at 15 ng/ml for three days to obtain an enriched population of monocytes. The monocytes were plated at a concentration 1000 cells per well in 96-well plates and incubated with RANKL (#390-TN-010, R&D Systems) and M-CSF (#216-MC-005, R&D Systems) at 15 ng/ml for both supplements for a period of 6 days, and Losartan were added to different plates at variable concentrations along with RANL and MCSF. During the osteoclast differentiation, the growth medium was changed every 48 h and the osteoclasts were stained for Tartrate-resistant acid phosphatase (TRAP) on day 6. TRAP staining was performed following the standard protocol: fixing the osteoclasts with 4% paraformaldehyde in PBS for 1 min at room temperature then staining for TRAP for 15 min at 37 degree (Acid Phosphatase, Leukocyte (TRAP) Kit, Sigma-Aldrich). Mature osteoclasts that were TRAP positive with three or more nuclei were counted using a standard light microscope.

RAW 264.7, a murine macrophage cell line, was kindly provided by Dr. Bryant Darnay (Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA) [19]. It was selected by limited dilution to express the highest osteoclastogenic potential. This clone (clone #28) has been used as an osteoclastogenic cell system that could differentiate into functional TRAP-positive osteoclasts in the presence of RANKL [20].

RAW 264.7 cells were cultured in 6-well plates at a density of $5 \times 10\text{^}4$ cells per well in DMEM/F12 supplemented with 10% fetal bovine serum and antibiotics and cultured overnight. On the second day, the medium was with DMEM/F12 without fetal bovine serum to starve the cells for 24 h. On the third day we replaced the medium with DMEM/F12 with Losartan (100 μM) or with vehicle (distilled water) for 1 h to saturate the binding of Losartan to its receptors. Then, we added either Angiotensin II (1 μM) or RANKL (100 nM) to stimulate the cells and collected the cell lysis at time points: 0, 10, 30 and 60 min afterwards by applying 2× laemmli buffer. The cell lyses were used for further western blot analysis.

To examine the ERK1/2 phosphorylation status, western Blot was performed following standard protocol. Phospho-ERK1/2 (#4370S,