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Impairing effects of angiotensin-converting enzyme inhibitor Captopril on bone of normal mice

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ABSTRACT

There are contradicting results about the effects of angiotensin-converting enzyme inhibitors (ACEIs) on bones. This study was aimed to investigate the effect of ACEI, Captopril, on bone metabolism and histology as well as the action of Captopril on skeletal renin–angiotensin system (RAS) and bradykinin receptor pathway in normal male mice. The urine, serum, tibias and femurs from normal control mice and Captopril-treated (10 mg/kg) mice were collected for biochemical, histological and molecular analyses after drug administration for eight weeks. The mice after the treatment with Captopril had a significant decrease of serum testosterone level. The histological measurements showed the loss of trabecular bone mass and trabecular bone number, and the breakage of trabecular bone network as well as the changes of chondrocyte zone at epiphyseal plate in Captopril-treated mice. The defect of Captopril on trabecular bone was reflected by the quantitative bio-parameters from micro-CT. The expression of renin receptor and bradykinin B2 receptor (B2R) was significantly up-regulated in tibia of mice upon to the Captopril treatment, which decreased the ratio of OPG/RANKL and the expression of osteoblastic factor RUNX2. Furthermore, Captopril treatment resulted in the increase of pAkt/Akt and pNFκB expression in tibia. The present study revealed the impairing effects of Captopril on bone via interfering with the circulating sex hormone level and B2R pathway, which suggests that the bone metabolism of patients need to be carefully monitored when being prescribed for ACEIs.

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1. Introduction

The renin–angiotensin system (RAS) plays a central role in the control of blood pressure and fluid balance within the body (Namazi et al., 2011). In addition to the systemic RAS, the emerging evidences revealed the expression and function of RAS in local tissue, namely tissue RAS, which is postulated to participate in various pathophysiologic processes (Inaba et al., 2011; Koïtka et al., 2010; Lau et al., 2004; Wong et al., 2010; Yung et al., 2011).

Recent in vivo studies showed the expression of RAS components in trabecular bone (Asaba et al., 2009; Izu et al., 2009; Zhang et al., 2014a, 2014b), and in vitro study demonstrated the existence of angiotensin receptors in primary osteoblasts (Asaba et al., 2009), indicating the RAS components are expressed locally in bone microenvironment. The animal studies in our group demonstrated that the skeletal RAS was involved in osteoporosis induced by ageing (Gu et al., 2012a), and trabecular bone injuries of mice with either obstructive nephropathy (Gu et al., 2012b) or type 1 diabetes (Diao et al., 2014). Furthermore, the other studies identified the roles of local bone RAS in fracture healing (Garcia et al., 2010), the steroid-induced osteonecrosis (Zhang et al., 2014a), and the development of postmenopausal osteoporosis in ovariectomized animal models (Liu et al., 2011; Shimizu et al., 2008) as well as glucocorticoid-induced osteoporosis (Shuai et al., 2015). Therefore, the previous studies suggested that the local RAS exists in bone tissue and has a vital biological action on bone metabolism.

The main effector peptide angiotensin II (Ang II) in RAS is produced from Ang I by the action of angiotensin-converting enzyme (ACE), a key molecule in RAS. ACE inhibitor (ACEI) could increase bone mineral density (BMD) and reduce fracture risk in patients (García-Testal et al., 2006; Lynn et al., 2006; Rejnmark et al., 2006). However, some clinical results showed that the application of ACEI did not have positive effects on bones (Stimpel et al., 1995), and even led to bone loss (Kwok et al., 2012; Masunari et al., 2008; Zhang et al., 2012b).

In animal studies, the ACEI treatment suppressed the estrogen deficiency-induced decrease in bone density (Shimizu et al., 2009),

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accelerated bone healing and remodeling (Garcia et al., 2010), and improved osteoporosis and hypertension (Asaba et al., 2009). While, our group recently elucidated that the treatment with ACEI, Captopril, significantly elevated the level of tartrate-resistant acid phosphatase in serum, and had a trend to decrease BMD and damage micro-architecture of trabecular bone in type 1 diabetic mice (Diao et al., 2014).

In view of the contradictory effects of ACEI on bone health, we are keen to know the effects of ACEI on bone tissue of normal mice at normotensive condition. This study was performed to investigate the effect of ACEI, Captopril, on bone mass, micro-architecture and histology, and the action of Captopril on skeletal RAS and bone metabolic regulators in normal male mice.

2. Materials and methods

2.1. Animal treatment

The 10-week-old male ICR mice (Slac Laboratory Animal, Shanghai, China) were randomly divided into normal control group ($n=7$) and Captopril-treated group (10 mg/kg, i.g, $n=8$). After the treatment for eight weeks, mean arterial blood pressure (MAP) was analysed by cannulation of the right carotid artery (Servomed, Hellige GmbH, Freiburg, Germany). The urine, serum, tibias and femurs were harvested for the biochemical, histological and molecular analyses. The protocol for animal study was reviewed and approved by the institution's Animal Ethics Committee at the Nantong University. Animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (8th edition, Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences, Washington DC).

2.2. Serum and urine chemistries

The concentrations of calcium (Ca), phosphorus (P) and creatinine (Cr) in serum and urine were measured by standard colorimetric methods using a micro-plate reader (Bio-Tek, USA). The urinary level of Ca and P was corrected by the concentration of urine Cr. The serum levels of bone turnover markers, tartrate-resistant acid phosphatase 5b (TRAP), and procollagen type I N-terminal propeptide (PINP) were determined using sandwich ELISA kit purchased from Immunodiagnostic Systems Ltd (Boldon, UK). The kit for serum testosterone was provided by ALPCO (USA).

2.3. Histological staining

The femurs were fixed in 4% formaldehyde/PBS (pH 7.2), decalcified in 0.5 M EDTA (pH 8.0), and embedded in paraffin by standard histological procedures. Serial sections of 3 μm were cut. Safranin O (Sigma-Aldrich) staining was performed, combining with fast green and counter stain by hematoxylin. Additionally, the Masson-Trichrome staining was also performed. Stained slides were visualized under microscope.

2.4. Tartrate-resistant acid phosphatase staining

Tartrate-resistant acid phosphatase (TRAP) staining was used for the identification of osteoclasts following the manufacturer's instructions (Sigma 387-A, St Louis, USA).

2.5. Micro-CT analysis

The femur without decalcification was fixed in a cylindrical plastic tube to prevent movement of the limb during measurement, and was scanned to obtain image. The distal femoral metaphysis was examined on 1.81 mm slab, corresponding to 173 slices, with a high-resolution micro viva-CT40 system (Scanco Medical, Bassersdorf, Switzerland). Trabecular bone was determined by a fixed threshold. The metaphyseal region of interest and trabecular compartments were isolated by hand-drawn contours based on 100 consecutive slices. The micro-architecture of trabecular bone was assessed with direct three-dimensional (3D) methods by μCT Evaluation Program (Image Processing Language v. 5.0A, Scanco). The 3D parameters for morphology and structure of trabecular bone were obtained as the following: (1) bone volume over total volume (BV/TV); (2) connectivity density (Conn.D); (3) structure model index (SMI); (4) trabecular bone number (Tb.N); (5) trabecular bone thickness (Tb.Th); (6) trabecular bone separation (Tb.Sp); (7) the mean mineral density of total volume (BMD/TV); (8) bone surface over bone volume (BS/BV).

2.6. RT-PCR

The tibia RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, California, USA). The synthesis of complementary DNAs (cDNAs) was performed by reverse transcription reactions with 4 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, California, USA) with oligo $dT_{(15)}$ primers (Fermentas) as described by the manufacturer. The obtained cDNAs served as the template for the regular polymerase chain reaction using a DNA Engine (ABI). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β2-microglobulin (β2-M) as housekeeping gene was used to determine the relative expression of the target genes. The primer sequence used in this study was as previously described (Gu et al., 2012a).

2.7. Western blotting

The proteins from tibia were extracted in Laemmli buffer, followed by 5 min boiling and centrifugation to obtain the protein lysis solution. After the determination on protein concentration by Bradford kits (Bio-Rad Laboratories, Hercules, CA, USA), 40 μg of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with 5% (w/v) nonfat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with one of the following primary antibodies at dilutions ranging from 1:500 to 1:200 at 4 °C overnight: mouse anti-renin monoclonal antibody, goat anti-angiotensin II polyclonal antibody, goat anti-bradykinin B1R polyclonal antibody, goat anti-bradykinin B2R polyclonal antibody, goat anti-Akt1/2 polyclonal antibody, rabbit anti-pAkt1/2/3 polyclonal antibody, and rabbit anti-pNFκB polyclonal antibody. All the above primary antibodies were purchased from Santa Cruz Biotechnology (USA). After three washes with TBST, membranes were incubated with secondary immunoglobulins conjugated to IRDye 800CW Infrared Dye (LI-COR), including donkey anti-goat, anti-mouse and anti-rabbit IgG with the dilution of 1:15000. Blots were visualized by the Odyssey Infrared Imaging System (LI-COR Biotechnology, USA) and densitometrically assessed on target bands with Odyssey Application Software (version 3.0). The internal reference protein β-actin was used for correcting unequal loading using the mouse monoclonal anti-β-actin antibody (Sigma, USA).

2.8. Statistical analysis

The data in this study were reported as mean ± standard error of mean (S.E.M.). All statistical analyses were performed using

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