Renin inhibitor aliskiren exerts beneficial effect on trabecular bone by regulating skeletal renin-angiotensin system and kallikrein-kinin system in ovariectomized mice

Yan Zhang [1,*#], Liang Wang [2,#], Yan Song [1], Xi Zhao [1], Man-Sau Wong [3], Wei Zhang [1]

[1] School of Pharmacy, Nantong University, Nantong 226001, China.

[2] Department of Orthopaedics, The 309th hospital of Chinese People's Liberation Army, Beijing 100091, China.

[3] Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong.

* Correspondence to: Dr. Yan Zhang, School of Pharmacy, Nantong University, Nantong 226001, Jiangsu Province, P.R.China.

Tel: 86-0513-85051728; Fax: 86-0513-85051730; E-mail: riceandtiger@163.com

Postal address: Qixiu Road 19, Nantong 226001, Jiangsu Province, China.

# contributed equally to this work.

This work was supported by National Natural Science Foundation of China (No. 81202894).

Summary

The skeletal renin-angiotensin system contributes to the development of osteoporosis. The renin inhibitor aliskiren exhibited beneficial effects on trabecular bone of osteoporotic mice, and this action might be mediated through angiotensin and bradykinin receptor pathways. This study implies the potential application of renin inhibitor in the management for postmenopausal osteoporosis.

Abstract

Purpose The skeletal renin-angiotensin system plays key role in the pathological process of osteoporosis. The present study is designed to elucidate the effect of renin inhibitor aliskiren on trabecular bone and its potential action mechanism in ovariectomized (OVX) mice.

Methods The OVX mice were treated with low dose (5 mg/kg), or high dose (25 mg/kg) of aliskiren or its vehicle for 8 weeks. The bone turnover markers were measured by ELISA. The structural parameters of trabecular bone at lumbar vertebra (LV) and distal femoral metaphysis were measured by micro-CT. The expression of mRNA and protein was studied by RT-PCR and immunoblotting, respectively.

Results Aliskiren treatment reduced urinary excretion of calcium and serum level of tartrate-resistant acid phosphatase in OVX mice. The treatment with aliskiren significantly increased bone volume (BV/TV) and connectivity density (Conn.D) of trabecular bone at LV-2 and LV-5 as well as dramatically enhanced BV/TV, Conn.D, bone mineral density (BMD/BV) and decreased bone surface (BS/BV) at the distal femoral end. Aliskiren significantly down-regulated the expression of angiotensinogen, angiotensin II (Ang II), Ang II type 1 receptor, bradykinin receptor (BR)-1, and osteocytic-specific gene sclerostin as well as the osteoclast-specific genes, including carbonic anhydrase II, matrix metalloproteinase-9, and cathepsin K.

Conclusions This study revealed that renin inhibitor aliskiren exhibited the beneficial effects on trabecular bone of ovariectomy-induced osteoporotic mice, and the underlying mechanism for this action might be mediated through Ang II and BR

signaling pathways in bone.

Keywords Aliskiren · Renin-angiotensin system · Kallikrein-kinin system · Trabecular bone · Ovariectomized

Introduction

The renin-angiotensin system (RAS) is a hormonal cascade that is thought to act as a master controller of blood pressure and fluid balance within the body [1]. Within classical RAS, liver secreted angiotensinogen (AGT) is enzymatically cleaved to angiotensin (Ang) I by kidney-derived renin. Ang I is, hereafter, cleaved by angiotensin-converting enzyme (ACE) to the effector hormone Ang II. It is now evident that the components of RAS, in addition to the classical pathway, are expressed and act locally in multiple tissues [2], such as insulin secretion [3], glomerular sclerosis [4], renal inflammation [5], atherosclerosis [6], cardiac hypertrophy [7], brain disorders [8], and follicular development and endometrial cancer in female reproductive tract [9].

Previous studies have shown the expression of renin in bone marrow cells and ACE in osteoblasts and osteoclasts [10, 11]. In bone tissue, Ang II is generated locally by endothelial cells [12] and also produced in the interstitial space [13], and then exerts its action by binding to angiotensin type 1 receptor (AT1R) and AT2R, both of which are expressed in osteoblasts [10, 14]. Functional studies revealed that Ang II could stimulate the differentiation and activity of osteoclasts in vivo [14] and in vitro [15], and aggravate the loss of bone minerals in rats with osteoporosis induced by estrogen deficiency [14], furthermore, the AT1R knockout mice showed high bone mass [16]. In addition, we recently demonstrated that the local RAS in bone was involved in age-related osteoporosis of aging mice [17], bone deteriorations of mice with either obstructive nephropathy [18] or type 1 diabetes [19], and others elucidated the

involvement of skeletal RAS in the process of fracture healing in a mouse femur fracture model [20] and the steroid-induced osteonecrosis in rabbits [21] as well as the development of postmenopausal osteoporosis in ovariectomized (OVX) animal models [14, 22]. Therefore, the emerging evidences demonstrated that the local RAS displays important biological actions in bone tissue.

Currently, besides the applications in the prevention and treatment of hypertension, the inhibitors of RAS [ACE inhibitors (ACEI), Ang II receptor blockers (ARB), and renin inhibitors] are widely used in the clinic to treat tissue injury due to locally high RAS activity, such as the renal and cardiovascular diseases [2]. The use of ARB/ACEI was reported to be associated with reduction of fracture risk [23]. The experimental studies have shown the beneficial effects of ACEI and ARB on maintaining bone health of OVX rats [14, 24, 25] and mice [11, 26], an animal model mimicking postmenopausal osteoporosis due to the decline of circulating estrogen level. While, given renin is the rate-limiting enzyme of the RAS, whether the RAS inhibition by inhibiting renin activity exerts beneficial effects on postmenopausal osteoporosis is not known.

Postmenopausal osteoporosis is the most common type of osteoporosis that contributes to morbidity and mortality in millions of menopausal women worldwide [27], and the major clinical consequences of this disease are osteoporotic fractures of the upper extremity, spine, and hip [28]. Thus, we recently performed an animal study to address the effects of renin inhibitor, aliskiren, on trabecular bone of mice with postmenopausal osteoporosis induced by ovariectomy. The aim of the present study is

to elucidate the impact of renin inhibitor on trabecular bone of osteoporotic mice.

Materials and methods

Animal welfare and ethical statement

The procedures used in this study were as humane as possible and this article complies with the recommendations of ARRIVE. The animal study protocol was reviewed and approved by the institution's Animal Ethics Committee of the Nantong University (Permit number: TD14-P003). Thirty-two female C57BL/6J mice with three-month-old (Slac Laboratory Animal, Shanghai, China) were housed in environmentally controlled central animal facilities. The animals were kept in 22°C, light : dark (12 h : 12 h) conditions and fed with commercial diet and distilled water *ad libitum* during experimental period.

Animal study design

The mice were allowed to acclimate to their environment for 1 week before surgery, during which the mice were either dorsal ovariectomized (OVX) or sham-operated (Sham) under anesthetization with a mixture of ketamine:xylazine (80:10 mg/kg). Starting from 1 week post-surgery, the mice were divided into four groups with eight in each group: Sham-operated mice (Sham), OVX mice with vehicle treatment (OVX), OVX mice with orally administration of low dose of aliskiren (OVX+LA, 5 mg/kg) and high dose of aliskiren (OVX+HA, 25 mg/kg). Eight weeks after drug administration, the systolic blood pressure was measured by a noninvasive tail-cuff method using a Model BP-98A (Softron, Tokyo, Japan), and spot urine of each mouse

was collected. Serum, tibias, femurs and lumbar vertebras were immediately harvested for a variety of analyses.

Serum and urine chemistries

Calcium (Ca) and creatinine (Cr) concentrations of serum and urine were measured by standard colorimetric methods using a micro-plate reader (Bio-Tek, USA). The level of urine Ca was corrected by the concentration of urine Cr. 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 osteocalcin (OCN) and renin was provided by Immutopics (San Clemente, USA) and Thermo Scientific (Frederick, USA).

Micro-computed tomography (Micro-CT) scanning

The lumbar vertebras-2 (LV-2), LV-5 and femurs without sample preparation or decalcification was fixed in a cylindrical plastic tube to prevent movement of the bone during scanning, and scanned with a high-resolution micro vivaCT 40 system (Scanco Medical, Bassersdorf, Switzerland). The parameters for each single scan were 70 kVp of the X-ray and 1000 projections per 180°. Trabecular bone was determined by a fixed threshold. After images were captured (110 μA), 100 slices were established as the volume of interest. Trabecular bone was separated from cortical bone by free drawing regions of interests using the software provided with the scanner.

Trabecular bone structural images and parameters

Morphologic measurements of the trabecular bone for the 100 slices were reconstructed to obtain 3-dimensional images and quantitative parameters with μCT Evaluation Program: (1) bone volume over total volume (BV/TV); (2) bone surface over bone volume (BS/BV); (3) connectivity density (Conn.D); (4) bone mineral density over bone volume (BMD/BV).

Tartrate-resistant acid phosphatase staining

Tartrate-resistant acid phosphatase (TRAP) staining performed on LV-4 and the distal metaphysis of femur was used for the identification of osteoclasts following the manufacturer's instructions (Sigma 387-A, St Louis, USA).

RT-PCR

The tibia of each animal was crushed under liquid nitrogen condition and RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, California, USA). RNA integrity was verified by agarose gel electrophoresis. Synthesis of 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 first strand cDNAs served as the template for the regular PCR performed using a DNA Engine (ABI). Glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) as an internal control was used to normalize the data to determine the relative expression of the target genes. The PCR primers used in this study were as previously described [16, 17, 29].

Western blotting

The tibias were homogenized and extracted in Laemmli buffer (Boston Bioproducts, Worcester, MA, USA), followed by 5 min boiling and centrifugation to obtain the supernatant. Samples containing 40 μg of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation 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 antibodies at dilutions ranging from 1:1000 to 1:300 at 4℃ overnight: mouse anti-renin monoclonal antibody, goat anti-angiotensin II (Ang II) polyclonal antibody, mouse anti-Ang II type 1 receptor monoclonal antibody, and goat anti-Ang II type 2 receptor 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 IgG and donkey anti-mouse IgG with the dilution of 1:15000. After 2 h-incubation at room temperature, membranes were washed three times with TBST. Blots were visualized by the Odyssey Infrared Imaging System (LI-COR Biotechnology, USA). Signals were densitometrically assessed (Odyssey Application Software version 3.0) and normalized to the β-actin

signals to correct for unequal loading using the mouse monoclonal anti-β-actin antibody (Sigma, USA).

Statistical analysis

The data from these experiments were reported as mean ± standard error of mean (SEM) for each group. The statistical analysis was performed using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey's multiple comparison test as a post test to compare the group means if overall $P < 0.05$. The difference with P value of less than 0.05 was considered statistically significant.

Results

Systolic blood pressure and circulating renin level

The ovariectomized (OVX) mice were mostly normotensive. At the end of the treatment (week 8), the systolic blood pressure of aliskiren-treated OVX mice was slightly lower compared to the untreated OVX mice, but the difference was not statistically significant (Table 1). Additionally, the serum level of renin was comparable among all groups. Thus, aliskiren did not significantly affect blood pressure and the circulating renin level in this animal model.

Biochemical markers in serum and urine

To clarify the potential effects of renin inhibitor, aliskiren, on bone metabolism of OVX mice, the content of calcium in serum and urine and the level of bone turnover markers in serum, including osteocalcin (OCN), tartrate-resistant acid phosphatase (TRAP), and procollagen type I N-terminal propeptide (PINP), were measured (Table 1). Ovariectomy did not produce the significant changes of serum Ca, OCN, or PINP, but induced the higher level of urinary calcium ($P < 0.05$) and serum TRAP ($P < 0.05$) than those of Sham group. The treatment of OVX mice by aliskiren at both doses could decrease urinary calcium level, and the significant difference ($P < 0.05$) was obtained between the high dose treatment group and OVX group. The value of serum TRAP level was comparable between the two aliskiren-treated groups, but statistically lower than that of OVX group ($P < 0.05$). The treatment with aliskiren did not alter serum level of calcium, OCN or PINP of OVX mice.

Effects of aliskiren on trabecular bone at lumbar vertebra

To clarify the effects of aliskiren on trabecular bone at lumbar vertebra (LV) of OVX mice, the micro-computed tomography was applied to measure the bone biological parameters of LV-2 and LV-5, including connectivity density (Conn.D), bone volume over total volume (BV/TV). Ovariectomy alone resulted in the significant decrease ($P < 0.05$) of Conn.D (Fig. 1A) at LV-2 and LV-5 and of BV/TV (Fig. 1B) at LV-2. The administration with aliskiren at both doses could dramatically ($P < 0.05$) increase Conn.D and BV/TV at LV-2, and the low dose and high dose of aliskiren could enhance ($P < 0.05$) BV/TV and Conn.D of LV-5, respectively. Furthermore, the 3D images at LV-2 (Fig. 1C) showed the breakage and loss of trabecular bone in OVX group, and these micro-architectural changes were improved after treatment with aliskiren for 8 weeks. Thus, the 3D images obtained from each group were well consistent with the quantitative data.

Effects of aliskiren on trabecular bone at distal femoral end

To quantitatively analyze the effects of aliskiren on trabecular bone of long bone, the micro-computed tomography was applied to measure the bone biological parameters of trabecular bone at distal femoral end, including connectivity density (Conn.D), bone mineral density over bone volume (BMD/BV), bone surface over bone volume (BS/BV) and bone volume over total volume (BV/TV). The representative 2D (Fig. 2A) and 3D (Fig. 2B) images clearly showed the loss of trabecular bone mass and micro-architectural networks in OVX group when comparing with that in Sham group,

and the improvement of these pathological changes of OVX mice in response to aliskiren treatment. The quantitative results (Fig.2 C-F) on trabecular bone parameters showed the decrease of Conn.D ($P < 0.05$), BMD/BV ($P < 0.05$) and BV/TV ($P < 0.01$), and the increase of BS/BV ($P < 0.05$) in OVX group. Low dose of aliskiren could significantly reduce BS/BV ($P < 0.05$) and enhance BV/TV ($P < 0.05$), and aliskiren at high dose could dramatically improve these biological parameters of trabecular bone at distal femoral end of OVX mice.

Tartrate-resistant acid phosphatase (TRAP) staining

TRAP staining was used to investigate the influence of aliskiren on maturation of osteoclasts in OVX mice. In the Sham control group, few osteoclasts could be identified in the trabecular bone area of LV-4 (Fig. 1D, shown by red arrow) and the distal femoral end (Fig. 2G), whereas the distinct increase of TRAP-positive osteoclasts in this area was observed in vehicle-treated OVX group, and the treatment with aliskiren decreased the osteoclasts number at both bone sites as compared to those of OVX mice.

mRNA expression of RAS components in bone

To determine the involvement of bone RAS in the regulation of aliskiren on bone metabolism, the mRNA expressions of RAS components, including renin, angiotensinogen (AGT), angiotensin-converting enzyme (ACE), and two types of angiotensin II type 1 receptor (AT1Ra, AT1Rb) were measured in tibia (Fig. 3). The

mice post ovariectomy displayed the significant up-regulation of renin (Fig. 3B, $P < 0.05$), AGT ($P < 0.01$) and ACE ($P < 0.01$), while, after the treatment with aliskiren, the mRNA expression of AGT and AT1Ra was lower ($P < 0.05$) than those of OVX group. The renin, ACE and AT1Rb mRNA expression in bone of OVX mice were not affected by aliskiren with either dose.

Protein expression of RAS components in bone

Besides the mRNA expression, the protein expressions of RAS components, including renin, angiotensin II (Ang II), AT1R and AT2R, were measured in tibia (Fig. 3). In consistent with the mRNA expression results, as the down-stream product by the action of renin and ACE on AGT, the protein expression of the bioactive peptide Ang II in RAS was increased in OVX mice and decreased in aliskiren-treated OVX mice (Fig. 3D, $P < 0.05$). In addition, ovariectomy induced the decreased level of AT2R protein expression ($P < 0.05$), and the administration with aliskiren up-regulated AT2R protein expression ($P < 0.05$) and down-regulated AT1R protein expression ($P < 0.05$) in tibia of OVX mice.

Protein expression of B1R and MAS in bone

Besides the measurements on the expression of RAS components, the protein expression of MAS and bradykinin 1 receptor (B1R), the component in the kinin-kallikrein system, was determined (Fig. 4). The expression of B1R protein in tibia was higher in OVX group than that in Sham group ($P < 0.01$), and both the low

dose ($P < 0.05$) and the high dose ($P < 0.01$) of aliskiren treatment significantly decreased the B1R protein expression of tibia in OVX mice. As noted, there was no significant difference of MAS expression among experimental groups.

mRNA expression of osteoclastic and osteocytic markers

The homeostasis of bone metabolism is partially regulated by the function of osteoclasts. Thus, the mRNA expression of osteoclastic markers in tibia was determined for further explaining the influence of aliskiren on osteoclasts-involved resorptive activity (Fig. 5). The mRNA expression of CAII ($P < 0.05$), MMP9 ($P < 0.01$) and Cathepsin K ($P < 0.01$) was significantly up-regulated in vehicle-treated OVX mice. Both the treatments with aliskiren at low dose and high dose dramatically reversed these changes in OVX mice, suggesting the potential modulation of aliskiren on osteoclastic resorptive activity. Additionally, the mRNA expression of sclerostin (SOST), a secretory product of osteocytes, was also measured. The operation of ovariectomy significantly induced the up-regulation ($P < 0.01$) of SOST mRNA expression, which was markedly down-regulated after treatment with low ($P < 0.05$) and high dose ($P < 0.01$) of aliskiren in OVX mice.

Discussion

Aliskiren, the first renin inhibitor approved for clinical use, is a small molecule competitive inhibitor that specifically inhibits the enzymatic activity of renin [30]. It could effectively suppress the rate-limiting step within RAS cascade to reduce the production of Ang II, the active peptide with multi-activities involved in tissue injuries. The recent studies have demonstrated that aliskiren is able to attenuate the progression of nephropathy [31, 32] and cardiovascular diseases [33], and improve insulin resistance [34] in diabetic patients and animals. Given that Ang II, the central effector of the RAS, activates multiple pathways in skeleton to induce bone deteriorations, in this study we demonstrated that the inhibition of renin activity by aliskiren alleviated the damages of trabecular bone in OVX mice, confirming the crucial role of the renin-angiotensin cascade in the development of postmenopausal osteoporosis and the beneficial effect of renin inhibitor aliskiren in osteoporosis induced by estrogen deficiency.

Aliskiren slightly lowered blood pressure in OVX mice, but the effect was not statistically significant. This was consistent with previous observations that aliskiren did not alter blood pressure in normotensive subjects [35], mice [32] and rats [36] (ovariectomy-induced osteoporotic mice were normotensive shown in this study), also in agreement with recent reports which demonstrated the blood-pressure independent protective effect of aliskiren on tissue injuries [37-39]. Moreover, the serum level of renin was not changed in OVX mice upon to aliskiren treatment. Thus, the therapeutic effect of aliskiren is unlikely by acting on systemic blood pressure and renin

production in circulation.

Renin is the rate-limiting enzyme of the RAS cascade. As such, it is considered as an ideal drug target for RAS blockade. The development of direct renin inhibitors (e.g. aliskiren), however, is much slower than that of angiotensin-converting enzyme (ACE) inhibitors (ACEI) and Ang II receptor blockers (ARB) [40]. Blockade of the RAS with ACEI and ARB inevitably disrupts the negative feedback loop that is critical for maintaining renin homeostasis, leading to compensatory induction of renin [5, 41]. This is the main cause accounting for that the treatment with losartan (belongs to ARB) alone could not exert beneficial effects on diabetes-induced osteoporosis [42] and hyperglycemia-induced renal disease [5] in type 1 diabetic mice. The emerging clinical evidences indicated that the uses of ACEI did not have beneficial effects on bones [43], and even led to bone loss in older American men [44], Chinese women [41] and Japanese [45]. Thus, whether aliskiren could therapeutically manage bone metabolism of OVX mice with experimentally estrogen deficiency-induced osteoporosis is the aim of this study.

The present study showed that the treatment with renin inhibitor aliskiren could effectively improve ovariectomy-induced pathological changes of micro-architecture of trabecular bone at the lumbar vertebra (LV)-2 and LV-5 including Conn.D and BV/TV, and at the distal femoral end including Conn.D, BV/TV, BS/BV and BMD/BV. The 2-dimensional (2D) and 3D images of the trabecular bone also consistently displayed that the renin inhibitor could recover the trabecular bone network as well as raise the bone mass and bone connectivity in osteoporotic mice

after ovariectomy surgery. These results indicated the preventive effects of inhibiting renin activity on loss of bone minerals and damages of trabecular bone structure at spine bone and long bone, while, whether there are similar effects of aliskiren on cortical bone of long bones (like the diaphysis of tibia and femur) need to be further clarified.

The status of bone metabolism in vivo could be reflected by the bone turnover biomarkers in serum and urine. The OVX mice after treatment with aliskiren in this study displayed the decreased bone resorption as demonstrated by the reduced excretion of urinary calcium and the decreased level of serum TRAP, moreover, aliskiren could markedly reduce the TRAP-positive osteoclasts number in trabecular bone and down-regulate the expression of osteoclast-specific genes, including carbonic anhydrase II (CAII), matrix metalloproteinase (MMP)-9 and Cathepsin K. MMP-9 is the enzyme secreted from the ruffled border of osteoclasts to dissolve the organic components of bone, and CAII in osteoclasts is responsible for dissolving the bone inorganic substance [29]. Cathepsin K, one of proteolytic enzymes, degrades organic components in the bone matrix [46]. Therefore, the present study revealed that the inhibition of aliskiren on bone resorption via suppressing the osteoclastogenesis and the osteoclastic resorptive activity might be the potential mechanism for the beneficial effects of aliskiren on trabecular bone of OVX mice.

It is well known that the high activity of skeletal RAS, especially the increased production of Ang II, the bioactive peptide within RAS, would lead to bone injuries [10, 11, 14-16]. This study showed that the ovariectomy alone increased the mRNA

expression of renin, AGT and ACE, consequently increased the Ang II protein expression, in accordance with the recent finding that the mRNA expression of renin and the protein expression of Ang II were up-regulated in tibia of rat with hyperglycemia [47]. After the treatment with aliskiren, the expression of AGT and Ang II was almost recovered to the level of Sham group. Furthermore, the present results showed the aliskiren-induced down-regulation of sclerostin (SOST), which is expressed chiefly on bone cells (osteocytes) and functionally inactivates Wnt signaling pathway, thereby SOST is a physiological inhibitor of bone formation [48]. The regulation of aliskiren on SOST expression was, at least partially, attributed to its down-regulating effect on AT1Ra mRNA expression and AT1R protein expression, as the AT1Ra-deficienct mice displayed a decreased expression of SOST in bone [16]. Thus, this study fully suggested the key role of skeletal RAS in estrogen deficiency-induced osteoporosis and provided a choice to target skeletal RAS, especially the renin activity, when performing the drug discovery for anti-postmenopausal osteoporosis.

Several publications raised the possibility that AT1R and AT2R carry out negative cross-talk within fibroblasts and vascular endothelial cells with respect to each other's signaling pathways and responses [49]. This study revealed that the expression of AT2R was lower in OVX mice than that in Sham mice, which was consistent with that AT2R is the protective arm of RAS and counterbalances pathological processes and enable recovery from disease [50, 51]. Of importance, the oppose regulation of aliskiren on AT1R and AT2R showed the unique characteristic of renin inhibitor,

which contributed to its protective effects against bone deteriorations by interacting with Ang II signaling pathways.

Besides the RAS, bradykinin, the major effector peptide of the kallikrein-kinin system (KKS), could increase osteoclastic formation, consequently stimulate bone resorption and reducing BMD through acting on the seven transmembrane G protein-coupled receptor, named B1R [52, 53]. The present study showed that aliskiren decreased the protein expression of B1R in OVX mice, indicating the use of aliskiren might reduce the B1R-involved signals of bradykinin in bone, thereafter locally improve bone metabolism. While the underlying mechanism for the modulation of aliskiren on KKS need to be further elucidated.

Taken together, the present study demonstrated that the trabecular bone was acted by renin inhibitor aliskiren besides its widely reported targeting tissues like heart, kidney, vascular and brain. Importantly, our study revealed the beneficial effects of aliskiren on trabecular bone of osteoporotic mice induced by estrogen deficiency, suggesting its potential application in clinic to manage postmenopausal osteoporosis. The underlying pharmacological mechanism may, at least partially, due to the regulation of aliskiren on osteoclast-involved bone resorptive activity, via acting on the Ang II and bradykinin signaling pathways in skeletal RAS and KKS, respectively. The potential efficacy of renin inhibitor on improving bone metabolism for postmenopausal women, especially those associated with hypertension, need to be further elucidated.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81202894).

Conflict of interest

Yan Zhang, Liang Wang, Yan Song, Xi Zhao, Man-Sau Wong, and Wei Zhang declare that they have no conflict of interest.

References

[1] Namazi S, Ardeshir-Rouhani-Fard S, Abedtash H (2011) The effect of renin angiotensin system on tamoxifen resistance. Med Hypotheses 77: 152-155.

[2] Skov J, Persson F, Frøkiær J, Christiansen JS (2014) Tissue Renin-Angiotensin systems: a unifying hypothesis of metabolic disease. Front Endocrinol (Lausanne) 5: 23.

[3] Lau T, Carlsson PO, Leung PS (2004) Evidence for a local angiotensin-generating system and dose-dependent inhibition of glucose-stimulated insulin release by angiotensin II in isolated pancreatic islets. Diabetologia 47: 240-248.

[4] Zhang Z, Zhang Y, Ning G, Deb DK, Kong J, Li YC (2008) Combination therapy with AT1 receptor blocker and vitamin D analog markedly ameliorates diabetic nephropathy. Proc Natl Acad Sci USA 105: 15896-15901.

[5] Zhang Y, Deb DK, Kong J, Ning G, Wang Y, Li G, Chen Y, Zhang Z, Strugnell S, Sabbagh Y, Arbeeny C, Li YC (2009) Long-term therapeutic effect of vitamin D analog Doxercalciferol on diabetic nephropathy: strong synergism with AT1 receptor antagonist. Am J Physiol Renal Physiol 297: F791-F801.

[6] Koïtka A, Cao Z, Koh P, Watson AMD, Sourris KC, Loufrani L, Soro-Paavonen A, Walther T, Woollard KJ, Jandeleit-Dahm KA, Cooper ME, Allen TJ (2010) Angiotensin II subtype 2 receptor blockade and deficiency attenuate the development of atherosclerosis in an apolipoprotein E-deficient mouse model of diabetes. Diabetologia 53: 584-592.

[7] Inaba S, Iwai M, Furuno M, Kanno H, Senba I, Okayama H, Mogi M, Higaki J,

Horiuchi M (2011) Role of angiotensin-converting enzyme 2 in cardiac hypertrophy induced by nitric oxide synthase inhibition. J Hypertens 29: 2236-2245.

[8] Naffah-Mazzacoratti Mda G, Gouveia TL, Simões PS, Perosa SR (2014) What have we learned about the kallikrein-kinin and renin-angiotensin systems in neurological disorders? World J Biol Chem 5: 130-140.

[9] Herr D, Bekes I, Wulff C (2013) Local renin-angiotensin system in the reproductive system. Front Endocrinol (Lausanne) 4: 150.

[10] Asaba Y, Ito M, Fumoto T, Watanabe K, Fukuhara R, Takeshita S, Nimura Y, Ishida J, Fukamizu A, Ikeda K (2009) Activation of renin-angiotensin system induces osteoporosis independently of hypertension. J Bone Miner Res 24: 241-250.

[11] Izu Y, Mizoguchi F, Kawamata A, Hayata T, Nakamoto T, Nakashima K, Inagami T, Ezura Y, Noda M (2009) Angiotensin II type 2 receptor blockade increases bone mass. J Biol Chem 284: 4857-4864.

[12] Lamparter S, Kling L, Schrader M, Ziegler R, Pfeilschifter J (1998) Effects of angiotensin II on bone cells in vitro. J Cell Physiol 175: 89-98.

[13] Kumar R, Boim MA (2009) Diversity of pathways for intracellular angiotensin II synthesis. Curr Opin Nephrol Hypertens 18: 33-39.

[14] Shimizu H, Nakagami H, Osako MK, Hanayama R, Kunugiza Y, Kizawa T, Tomita T, Yoshikawa H, Ogihara T, Morishita R (2008) Angiotensin II accelerates osteoporosis by activating osteoclasts. FASEB J 22: 2465-2475.

[15] Hiruma Y, Inoue A, Hirose S, Hagiwara H (1997) Angiotensin II stimulates the proliferation of osteoblast-rich populations of cells from rat calvariae. Biochem

Biophys Res Commun 230: 176-178.

[16] Kaneko K, Ito M, Fumoto T, Fukuhara R, Ishida J, Fukamizu A, Ikeda K (2011) Physiological function of the angiotensin AT1a receptor in bone remodeling. J Bone Miner Res 26: 2959-2966.

[17] Gu SS, Zhang Y, Li XL, Wu SY, Diao TY, Hai R, Deng H (2012) Involvement of the skeletal renin-angiotensin system in age-related osteoporosis of ageing mice. Biosci Biotechnol Biochem 76: 1367-1371.

[18] Gu SS, Zhang Y, Wu SY, Diao TY, Gebru YA, Deng H (2012) Early molecular responses of bone to obstructive nephropathy induced by unilateral ureteral obstruction in mice. Nephrology 17: 767-773.

[19] Diao TY, Pan H, Gu SS, Chen X, Zhang FY, Wong MS, Zhang Y (2014) Effects of angiotensin-converting enzyme inhibitor, captopril, on bone of mice with streptozotocin-induced type 1 diabetes. J Bone Miner Metab 32: 261-270.

[20] Garcia P, Schwenzer S, Slotta JE, Scheuer C, Tami AE, Holstein JH, Histing T, Burkhardt M, Pohlemann T, Menger MD (2010) Inhibition of angiotensin-converting enzyme stimulates fracture healing and periosteal callus formation - role of a local renin-angiotensin system. Br J Pharmacol 159: 1672-1680.

[21] Zhang Y, Wang K, Song Q, Liu R, Ji W, Ji L, Wang C (2014) Role of the local bone renin-angiotensin system in steroid-induced osteonecrosis in rabbits. Mol Med Rep 9: 1128-1134.

[22] Liu YY, Yao WM, Wu T, Xu BL, Chen F, Cui L (2011) Captopril improves osteopenia in ovariectomized rats and promotes bone formation in osteoblasts. J Bone

Miner Metab 29: 149-158.

[23] Yamamoto S, Kido R, Onishi Y, Fukuma S, Akizawa T, Fukagawa M, Kazama JJ, Narita I, Fukuhara S (2015) Use of renin-angiotensin system inhibitors is associated with reduction of fracture risk in hemodialysis patients. PLoS One 10: e0122691.

[24] Shimizu H, Nakagami H, Osako MK, Nakagami F, Kunugiza Y, Tomita T, Yoshikawa H, Rakugi H, Ogihara T, Morishita R (2009) Prevention of osteoporosis by angiotensin-converting enzyme inhibitor in spontaneous hypertensive rats. Hypertens Res 32: 786-790.

[25] Donmez BO, Ozdemir S, Sarikanat M, Yaras N, Koc P, Demir N, Karayalcin B, Oguz N (2012) Effect of angiotensin II type 1 receptor blocker on osteoporotic rat femurs. Pharmacol Rep 64: 878-888.

[26] Kang KY, Kang Y, Kim M, Kim Y, Yi H, Kim J, Jung HR, Park SH, Kim HY, Ju JH, Hong YS (2013) The effects of antihypertensive drugs on bone mineral density in ovariectomized mice. J Korean Med Sci 28: 1139-1144.

[27] Reginster JY, Burlet N (2006) Osteoporosis: a still increasing prevalence. Bone 38: S4-S9.

[28] Ghosh M, Majumdar SR (2014) Antihypertensive medications, bone mineral density, and fractures: a review of old cardiac drugs that provides new insights into osteoporosis. Endocrine 46: 397-405.

[29] Zhang Y, Dong XL, Leung PC, Wong MS (2009) Differential mRNA expression profiles in proximal tibia of aged rats in response to ovariectomy and low-Ca diet. Bone 44: 46-52.

[30] Wood JM, Maibaum J, Rahuel J, Grütter MG, Cohen NC, Rasetti V, Rüger H, Göschke R, Stutz S, Fuhrer W, Schilling W, Rigollier P, Yamaguchi Y, Cumin F, Baum HP, Schnell CR, Herold P, Mah R, Jensen C, O'Brien E, Stanton A, Bedigian MP (2003) Structure-based design of aliskiren, a novel orally effective renin inhibitor. Biochem Biophys Res Commun 308: 698-705.

[31] Persson F, Rossing P, Parving HH (2013) Direct renin inhibition in chronic kidney disease. Br J Clin Pharmacol 76: 580-586.

[32] Zhang Y, Wang Y, Chen Y, Deb DK, Sun T, Zhao Q, Li YC (2012) Inhibition of renin activity by aliskiren ameliorates diabetic nephropathy in type 1 diabetes mouse model. J Diabetes Mellitus 2: 353-360.

[33] Riccioni G (2013) The role of direct renin inhibitors in the treatment of the hypertensive diabetic patient. Ther Adv Endocrinol Metab 4: 139-145.

[34] Gandhi S, Srinivasan B, Akarte AS (2013) Aliskiren improves insulin resistance and ameliorates diabetic renal vascular complications in STZ-induced diabetic rats. J Renin Angiotensin Aldosterone Syst 14: 3-13.

[35] Nussberger J, Wuerzner G, Jensen C, Brunner HR (2002) Angiotensin II suppression in humans by the orally active renin inhibitor Aliskiren (SPP100): comparison with enalapril. Hypertension 39: E1-8.

[36] Koid SS, Ziogas J, Campbell DJ (2014) Aliskiren reduces myocardial ischemia-reperfusion injury by a bradykinin B2 receptor- and angiotensin AT2 receptor-mediated mechanism. Hypertension 63: 768-773.

[37] Desjarlais M, Dussault S, Dhahri W, Mathieu R, Rivard A (2015) Direct renin inhibition with aliskiren improves ischemia-induced neovascularization: Blood pressure-independent effect. Atherosclerosis 242: 450-460.

[38] Yong QC, Thomas CM, Seqqat R, Chandel N, Baker KM, Kumar R (2013) Angiotensin type 1a receptor-deficient mice develop diabetes-induced cardiac dysfunction, which is prevented by renin-angiotensin system inhibitors. Cardiovasc Diabetol 12:169.

[39] Moriya H, Kobayashi S, Ohtake T, Tutumi D, Mochida Y, Ishioka K, Oka M, Maesato K, Hidaka S, Nomura S (2013) Aliskiren, a direct renin inhibitor, improves vascular endothelial function in patients on hemodialysis independent of antihypertensive effect - a pilot study. Kidney Blood Press Res 37: 190-198.

[40] Li YC (2007) Inhibition of renin: An updated review of the development of renin inhibitors. Curr Opin Investig Drugs 8: 750-757.

[41] Zhang YF, Qin L, Leung PC, Kwok TC (2012) The effect of angiotensin-converting enzyme inhibitor use on bone loss in elderly Chinese. J Bone Miner Metab 30: 666-673.

[42] Zhang Y, Diao TY, Gu SS, Wu SY, Gebru YA, Chen X, Wang JY, Ran S, Wong MS (2014) Effects of angiotensin II type 1 receptor blocker on bones in mice with type 1 diabetes induced by streptozotocin. J Renin Angiotensin Aldosterone Syst 15: 218-227.

[43] Stimpel M, Jee WS, Ma Y, Yamamoto N, Chen Y (1995) Impact of antihypertensive therapy on postmenopausal osteoporosis: effects of the angiotensin

converting enzyme inhibitor moexipril, 17beta-estradiol and their combination on the ovariectomy-induced cancellous bone loss in young rats. J Hypertens 13: 1852-1856.

[44] Kwok T, Leung J, Zhang YF, Bauer D, Ensrud KE, Barrett-Connor E, Leung PC; Osteoporotic Fractures in Men (MrOS) Research Group (2012) Does the use of ACE inhibitors or angiotensin receptor blockers affect bone loss in older men? Osteoporos Int 23: 2159-2167.

[45] Masunari N, Fujiwara S, Nakata Y, Furukawa K, Kasagi F (2008) Effect of angiotensin converting enzyme inhibitor and benzodiazepine intake on bone loss in older Japanese. Hiroshima J Med Sci 57: 17-25.

[46] Kim KR, Kim HJ, Lee SK, Ma GT, Park KK, Chung WY (2015) 15-deoxy-δ12,14-prostaglandin j2 inhibits osteolytic breast cancer bone metastasis and estrogen deficiency-induced bone loss. PLoS One 10: e0122764.

[47] Li Y, Shen GS, Yu C, Li GF, Shen JK, Xu YJ, Gong JP (2015) Local bone interaction between renin-angiotensin system and kallikrein-kinin system in diabetic rat. Int J Clin Exp Pathol 8: 1604-1612.

[48] Roux S (2010) New treatment targets in osteoporosis. Joint Bone Spine 77: 222-228.

[49] Yayama K, Okamoto H (2008) Angiotensin II-induced vasodilation via type 2 receptor: role of bradykinin and nitric oxide. Int Immunopharmacol 8: 312-318.

[50] Horiuchi M, Iwanami J, Mogi M (2012) Regulation of angiotensin II receptors beyond the classical pathway. Clin Sci (Lond) 123: 193-203.

[51] Namsolleck P, Recarti C, Foulquier S, Steckelings UM, Unger T (2014) AT(2)

receptor and tissue injury: therapeutic implications. Curr Hypertens Rep 16: 416.

[52] Souza PP, Brechter AB, Reis RI, Costa CA, Lundberg P, Lerner UH (2013) IL-4 and IL-13 inhibit IL-1β and TNF-α induced kinin B1 and B2 receptors through a STAT6-dependent mechanism. Br J Pharmacol 169: 400-412.

[53] Srivastava S, Sharma K, Kumar N, Roy P (2014) Bradykinin regulates osteoblast differentiation by Akt/ERK/NFκB signaling axis. J Cell Physiol 229: 2088-2105.

Figure legends

Figure 1

Quantitative biological parameters (A, B) of the trabecular bone at lumbar vertebra (LV)-2 and LV-5, measured by micro-computed tomography, and the representative micro-computed tomography 3-dimensional images (C) of LV-2 as well as the tartrate-resistant acid phosphatase staining (D) of the trabecular bone at LV-4 were shown in Sham mice and OVX mice treated with vehicle (OVX) or low (LA, 5 mg/kg) or high (HA, 25 mg/kg) dose of renin inhibitor, aliskiren, for 8 weeks. Conn.D, connectivity density; BV/TV, bone volume over total volume. D, osteoclasts shown by arrows in red (magnification, ×100). Values are expressed as means ± SEM, n = 7~8. * $P < 0.05$, vs. Sham; # $P < 0.05$, ## $P < 0.01$, vs. OVX.

Figure 2

A representative microcomputed tomography 2- (A) and 3-dimensional (B) image, the quantitative biological parameters (C-F), and the tartrate-resistant acid phosphatase staining (G) of the trabecular bone at distal femoral end in Sham mice and OVX mice treated with vehicle (OVX) or low (LA, 5 mg/kg) or high (HA, 25 mg/kg) dose of renin inhibitor, aliskiren, for 8 weeks. Conn.D, connectivity density; BMD/BV, bone mineral density over bone volume; BS/BV, bone surface over bone volume; BV/TV, bone volume over total volume. G, osteoclasts shown by arrows in red (magnification, ×200). Values are expressed as means ± SEM, n = 7~8. * $P < 0.05$, ** $P < 0.01$, vs. Sham; # $P < 0.05$, vs. OVX.

Figure 3

mRNA expression of RAS components in tibia (A), and the densitometric quantification of the mRNA expression levels, which are expressed as a ratio to the expression of GAPDH (B). Protein expression of renin, angiotensin II (Ang II) and its receptors in tibia (C) and the densitometric quantification of the protein expression levels, which are expressed as a ratio to the expression of β-actin (D). AGT, angiotensinogen; ACE, angiotensin-converting enzyme. AT1R, Ang II type 1 receptor; AT2R, Ang II type 2 receptor. Values are expressed as means ± SEM, n = 6. * $P < 0.05$, ** $P < 0.01$, vs. Sham; # $P < 0.05$, vs. OVX.

Figure 4

Protein expression of bradykinin receptor-1 and MAS in tibia (A) and the densitometric quantification for Western blotting, which are expressed as a ratio to the expression of β-actin (B). Values are expressed as means ± SEM, n = 6. Values are expressed as means ± SEM, n = 6. ** $P < 0.01$, vs. Sham; # $P < 0.05$, ## $P < 0.01$, vs. OVX.

Figure 5

mRNA expression of osteoclastic- and osteocytic-specific genes in tibia (A), and the densitometric quantification of the mRNA expression levels, which are expressed as a ratio to the expression of GAPDH (B). CAII, carbonic anhydrase II; MMP9, matrix

metalloproteinase-9; SOST, sclerostin. Values are expressed as means ± SEM, n = 6.

* $P < 0.05$, ** $P < 0.01$, vs. Sham; # $P < 0.05$, ## $P < 0.01$, vs. OVX.

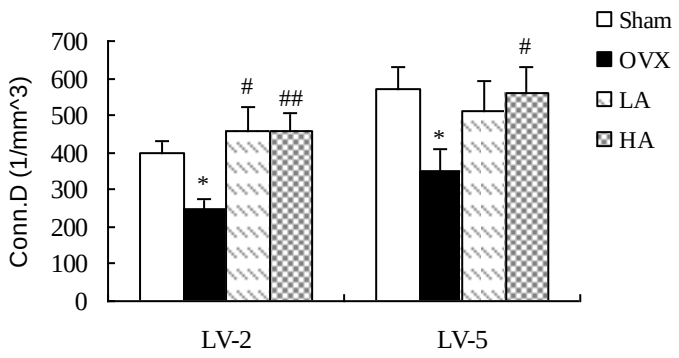
Table 1 Effects of aliskiren on blood pressure and biochemical markers in serum and urine of ovariectomized mice

	Serum Ca (mg/L)	Urine Ca/Cr (mg/mg)	Serum OCN (ng/ml)	Serum TRAP (U/L)	Serum PINP (ng/ml)	Serum Renin (ng/ml)	Blood Pressure (mmHg)
Sham	90.6 ± 3.5	0.119 ± 0.012	45.5 ± 6.1	9.23 ± 0.51	41.6 ± 2.0	10.8 ± 1.2	107 ± 4
OVX	87.7 ± 3.4	0.210 ± 0.036*	36.7 ± 3.2	11.25 ± 0.58*	40.2 ± 2.3	10.5 ± 1.3	111 ± 6
OVX+LA	88.5 ± 2.0	0.152 ± 0.031	40.0 ± 3.1	7.84 ± 0.33#	41.2 ± 1.2	11.0 ± 1.3	110 ± 3
OVX+HA	89.1 ± 3.3	0.106 ± 0.009#	48.0 ± 7.4	7.91 ± 0.41#	39.4 ± 2.0	9.9 ± 0.8	108 ± 5

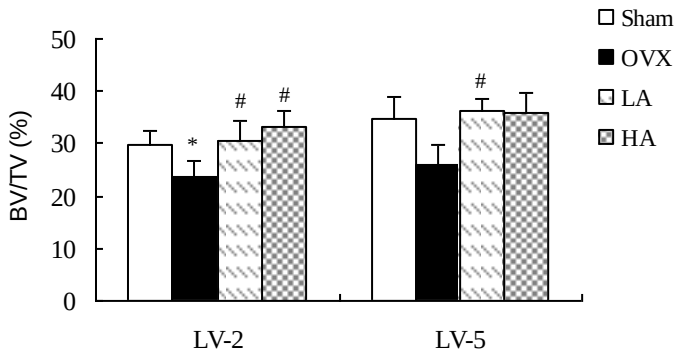
Sham and OVX mice were subjected to the following treatments for 8 weeks: Sham, Sham-operated mice with vehicle treatment; OVX, ovariectomized mice with vehicle treatment; OVX+LA, OVX mice with low dose of aliskiren (5 mg/kg); OVX+HA, OVX mice with high dose of aliskiren (25 mg/kg). Values are expressed as means ± SEM, $n = 8$ in each group. * $P < 0.05$, vs. Sham; # $P < 0.05$, vs. OVX. Ca, calcium; Cr, creatinine; OCN, osteocalcin; TRAP, tartrate-resistant acid phosphatase; PINP, procollagen type I N-terminal propeptide.

Figure 1

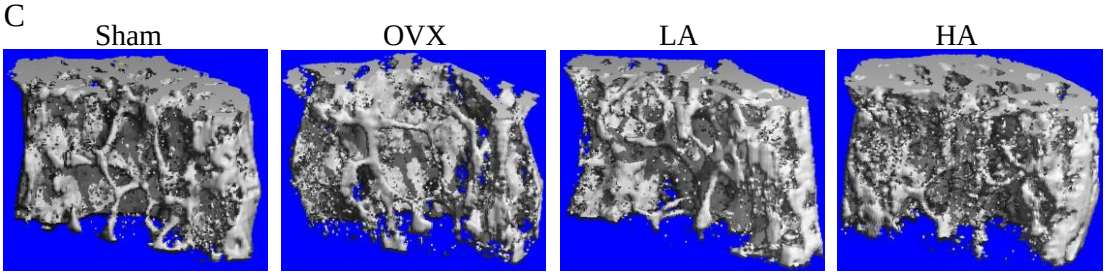
A



B



C



D

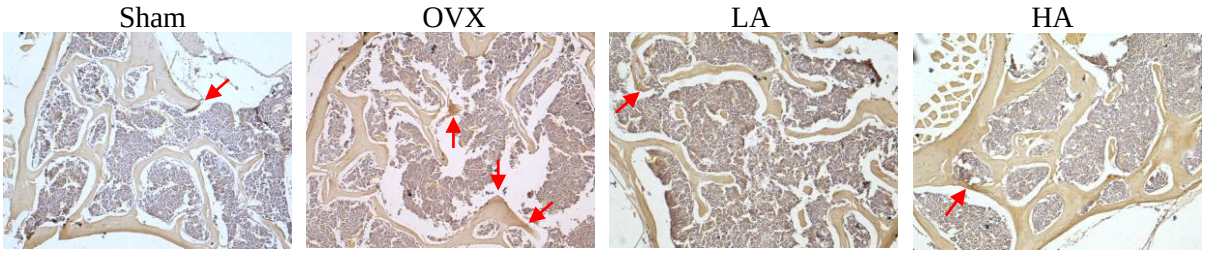


Figure 2

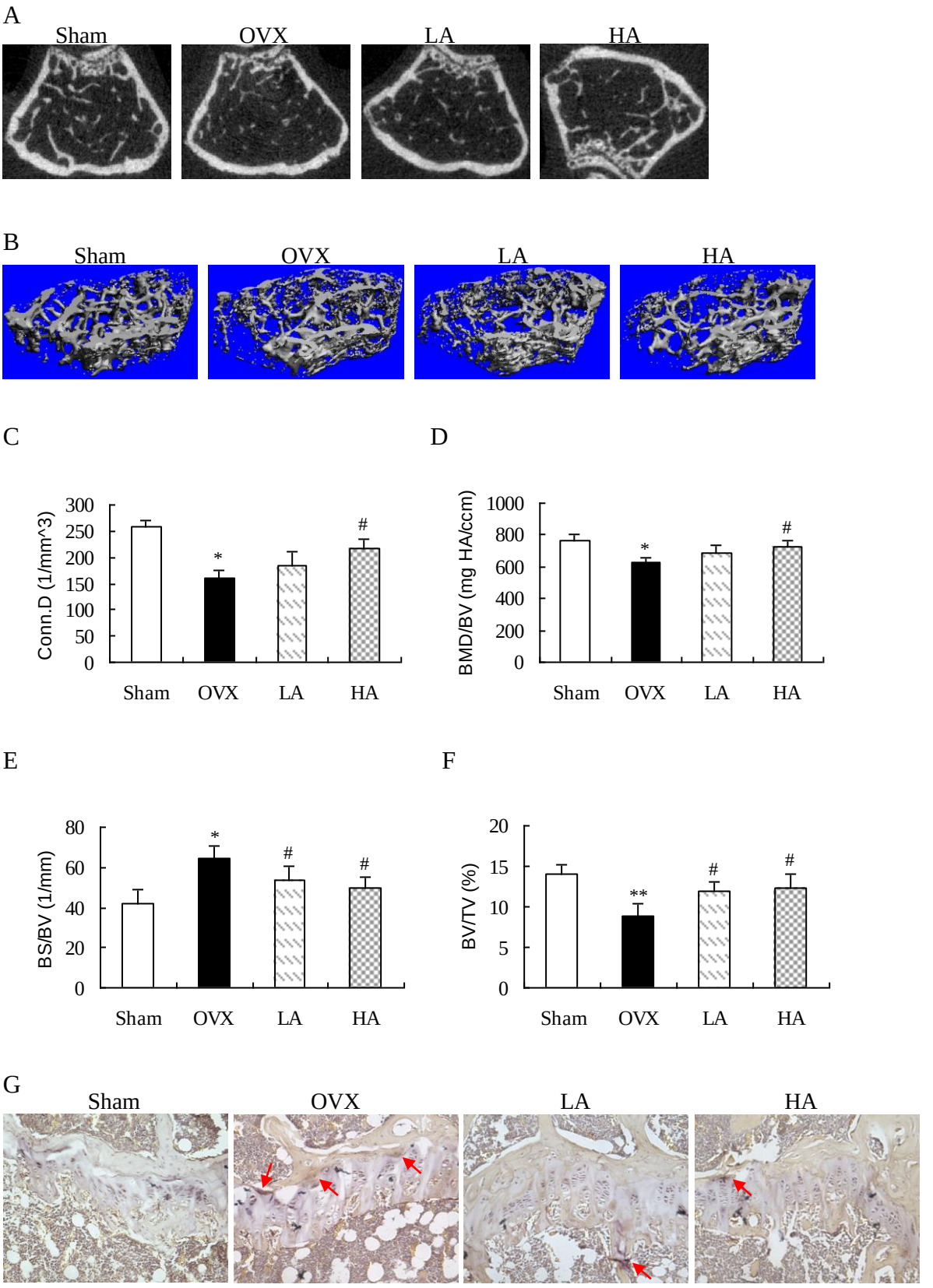

A
Sham
OVX
LA
HA
B
Sham
OVX
LA
HA
C
Conn.D (1/mm^3)
300
250
200
150
100
50
0
*
#
Sham
OVX
LA
HA
D
BMD/BV (mg HA/ccm)
1000
800
600
400
200
0
*
#
Sham
OVX
LA
HA
E
BS/BV (1/mm)
80
60
40
20
0
*
#
#
Sham
OVX
LA
HA
F
BV/TV (%)
20
15
10
5
0
**
#
#
Sham
OVX
LA
HA
G
Sham
OVX
LA
HA

Figure 3

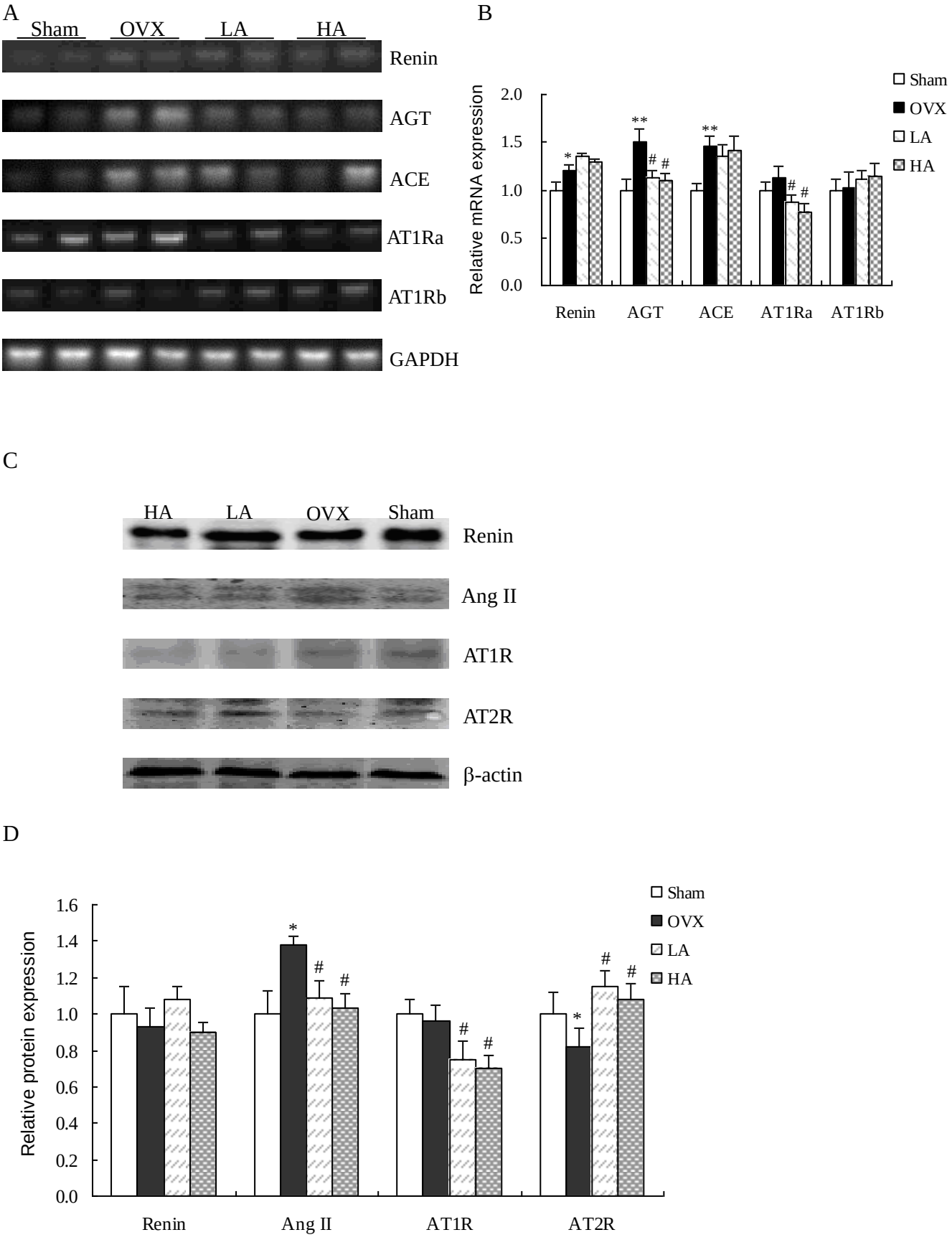
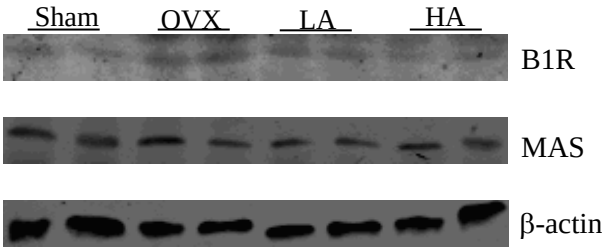

A
Sham
OVX
LA
HA
Renin
AGT
ACE
AT1Ra
AT1Rb
GAPDH
B
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
Sham
OVX
LA
HA
Renin
AGT
ACE
AT1Ra
AT1Rb
C
HA
LA
OVX
Sham
Renin
Ang II
AT1R
AT2R
β-actin
D
Relative protein expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham
OVX
LA
HA
Renin
Ang II
AT1R
AT2R

Figure 4

A


Sham
OVX
LA
HA
B1R
MAS
β-actin

B

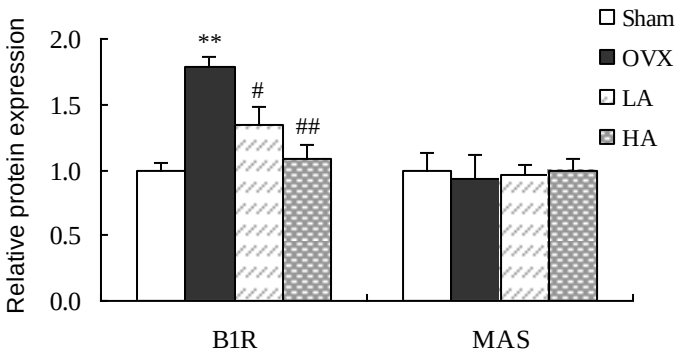
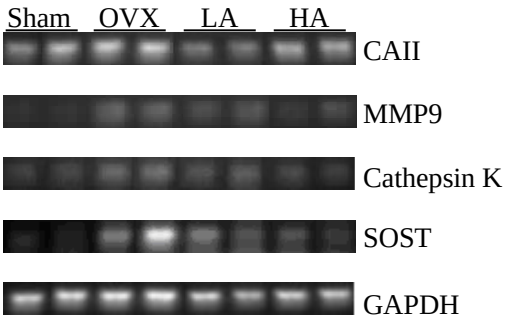
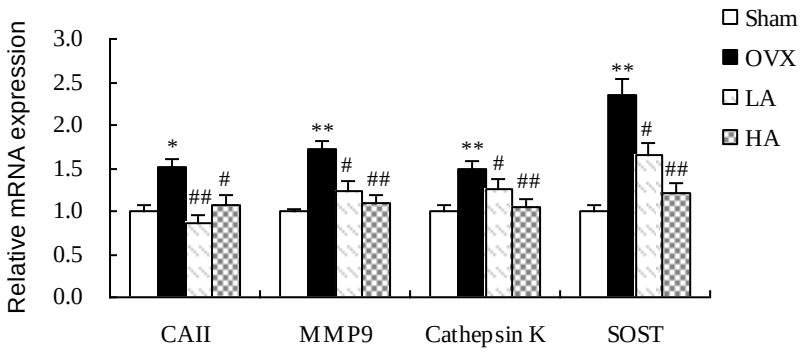

Relative protein expression
2.0
1.5
1.0
0.5
0.0
**
#
##
Sham
OVX
LA
HA
B1R
MAS

Figure 5

A


Sham
OVX
LA
HA
CAII
MMP9
Cathepsin K
SOST
GAPDH

B


Relative mRNA expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Sham
OVX
LA
HA
CAII
MMP9
Cathepsin K
SOST