

## ORIGINAL ARTICLE

# Prevention of osteoporosis by angiotensin-converting enzyme inhibitor in spontaneous hypertensive rats

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A recent analysis of clinical studies suggests that angiotensin-converting enzyme (ACE) inhibitors might reduce bone fractures. In this study, we examined whether an ACE inhibitor might attenuate osteoporosis in a hypertensive rat model. In spontaneous hypertensive rats (SHRs), estrogen deficiency induced by ovariectomy (OVX) resulted in a significant increase in osteoclast activation as assessed by the tartrate-resistant acid phosphatase (TRAP) activity in the tibia, accompanied by a significant decrease in bone density evaluated by dual-energy X-ray absorptiometry and an increase in urinary deoxypyridinoline. Treatment with an ACE inhibitor, imidapril, attenuated OVX-induced decrease in bone density and increase in TRAP activity and urinary deoxypyridinoline. As ACE inhibitors possess the effects of blockade of the renin–angiotensin system (RAS) and activation of the bradykinin–nitric oxide pathway, we examined the contribution of both pathways in an OVX-induced osteoporosis model. Administration of nitro-L-arginine methylester (L-NAME) did not alter TRAP activity, urinary deoxypyridinoline or bone density, whereas the administration of a subpressor dose of angiotensin II accelerated the increase in TRAP activity in the tibia, accompanied by a significant decrease in bone density and an increase in urinary deoxypyridinoline. Thus, ACE inhibitors prevented osteoporosis, probably because of the inhibition of RAS, but not of nitric oxide. Overall, ACE inhibitors attenuated osteoporosis in a hypertensive rat model through the blockade of RAS.

*Hypertension Research* (2009) 32, 786–790; doi:10.1038/hr.2009.99; published online 10 July 2009

**Keywords:** bone; renin–angiotensin system; osteoporosis

## INTRODUCTION

Hypertension and osteoporosis are two common diseases in the elderly population, and 50% of the hypertensive population comprises postmenopausal women at high risk of osteoporosis. Compared with healthy controls, reduced bone mineral density (BMD) has been found in patients with hypertension or ischemic heart disease.<sup>1–4</sup> Clinical studies have shown significant abnormalities in calcium metabolism in hypertension,<sup>5–7</sup> and a decreased circulating level of calcium results in an increased parathyroid hormone, which is implicated in the occurrence and/or development of hypertension.<sup>8</sup> Animal and epidemiological evidence suggests that high blood pressure is associated with abnormalities of calcium metabolism, leading to an increase in calcium loss, to a secondary activation of the parathyroid gland and to an increased movement of calcium from bone, thereby increasing the risk of osteoporosis.<sup>7,9</sup> Indeed, clinical studies have shown considerable evidence that antihypertensive drugs such as thiazides decreased the risk of hip fracture by reducing renal calcium excretion.<sup>10,11</sup> Interestingly, angiotensin-converting enzyme (ACE) inhibitors are also reported to reduce the risk of fractures.<sup>12–14</sup>

Recently, we reported that angiotensin (Ang) II activated osteoclasts, leading to osteoporosis.<sup>15</sup> As vasculature has an important role in bone remodeling, the effect of ACE inhibitors on bone metabolism may not only be a blockade of the renin–angiotensin system (RAS) but may also be related to the regulation of blood flow through the regulation of the bradykinin–nitric oxide pathway. Therefore, in this study, we examined the effect of an ACE inhibitor, imidapril, in an osteoporosis model in hypertensive rats, especially focusing on osteoclast activation.

## METHODS

### Rat ovariectomy osteoporosis model

All experiments were approved by the Ethical Committee for animal experiments at the Osaka University Graduate School of Medicine. Female adult Wistar rats (10-weeks old) were purchased from SLC Japan (Shizuoka, Japan). After the rats were anesthetized with intraperitoneal ketamine (80 mg kg<sup>-1</sup>) and xylazine (10 mg kg<sup>-1</sup>), bilateral ovariectomy (OVX) or sham operation was performed, and nitro-L-arginine methylester (L-NAME, Sigma, St Louis, MO, USA) was administered along with drinking water (1 mg ml<sup>-1</sup>).<sup>16</sup> Female adult

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Received 27 December 2008; revised 16 May 2009; accepted 2 June 2009; published online 10 July 2009

spontaneous hypertensive rats (SHRs) (10-weeks old) were also purchased from SLC Japan), and bilateral OVX or sham operation was performed. In some groups of rats, imidapril (1 or 3 mg kg<sup>-1</sup> per day) was administered along with drinking water. The body weight of these rats was recorded for 4 weeks. At 4 weeks after operation, systolic blood pressure was measured using the tail-cuff method (BP-98A, Softron, Koenji, Tokyo, Japan), and urinary deoxypyridinorine was measured by EIA (Metra Biosystems, Mountain View, CA, USA). They were strongly anesthetized and killed to collect femurs, tibias and blood for biochemical analysis.

#### Activity of ALP and tartrate-resistant acid phosphatase

Alkaline phosphatase (ALP) activity was measured for the evaluation of osteoblast activity, and tartrate-resistant acid phosphatase (TRAP) was measured for the evaluation of osteoclast activity, as previously described.<sup>15</sup> Proximal tibia and distal femur were excised and homogenized in 10 mmol l<sup>-1</sup> triethanolamine buffer (pH 7.5) for TRAP, and in diethanolamine buffer (pH 9.8) for ALP activity. Supernatants were subjected to a measurement of TRAP activity as previously described.<sup>17</sup> For ALP activity, supernatants were incubated with p-nitrophenylphosphate as a substrate for 30 min at 25 °C, and absorbance was measured at 405 nm and converted into unit (U: 1u indicates the release of 1 μmol p-nitrophenol per minute). The urinary deoxypyridinoline level was measured by EIA on day 28 of the experiment.

Serum calcium, phosphate, ALP and urinary calcium were measured in Falco Biosystems (Osaka, Japan) on day 28 of the experiment. Serum osteocalcin was

measured by EIA (Takara MK121 Rat Gla-OC Competitive EIA Kit, Otsu, Shiga, Japan).

#### Dual-energy X-ray absorptiometry

Bone density measurements were determined by dual-energy X-ray absorptiometry (DEXA) bone densitometry (GE-Lunar DPX-IQ, Madison, WI, USA). High- and low-beam energies for all scans were 80 and 35 kV, respectively, at 0.5 mA, as previously described.<sup>18</sup> BMD was obtained in g cm<sup>-2</sup>.

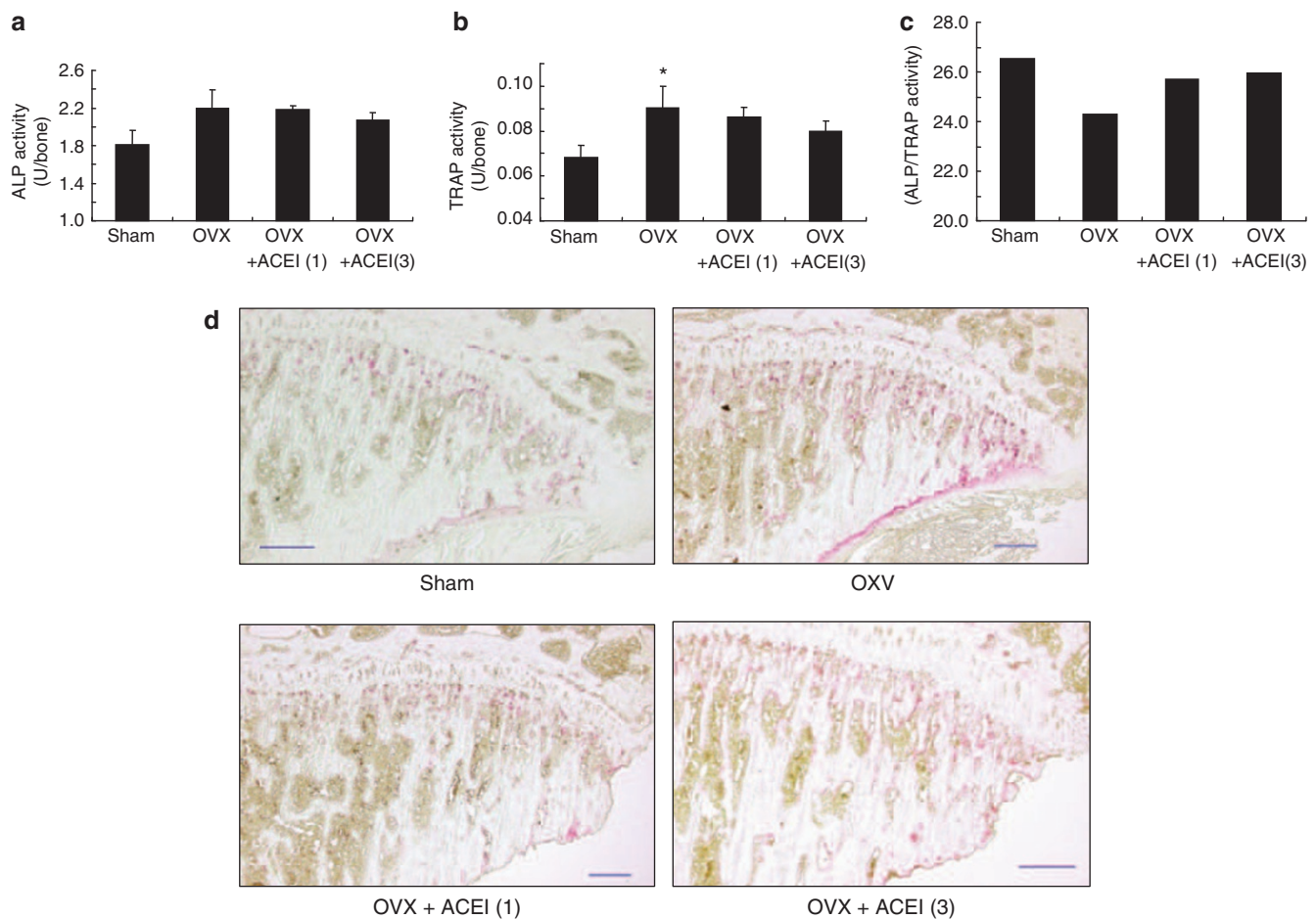
#### Statistical analysis

All values are expressed as mean ± s.e.m. Analysis of variance with subsequent Bonferroni's/Dunnett's test was used to determine the significance of differences in multiple comparisons. Values of *P* < 0.05 were considered to be statistically significant.

## RESULTS

### Imidapril ameliorated OVX-induced osteoporosis

To clarify the effect of ACE inhibition on bone metabolism, we used an OVX model of estrogen deficiency in a hypertension model, SHR.<sup>15</sup> At 28 days after bilateral OVX, both ALP and TRAP activities were increased in the tibia of ovariectomized SHR as compared with those in sham-operated SHR (Figures 1a and b). As TRAP activity was significantly increased, the ratio of ALP to TRAP was markedly



**Figure 1** Effects of angiotensin-converting enzyme (ACE) inhibitor, imidapril, on alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activity in ovariectomized spontaneous hypertensive rats (SHRs). (a) ALP activity, (b) TRAP activity, (c) ratio of ALP to TRAP activity, (d) TRAP staining after 28 days of each treatment. Sham=sham operation; OVX=bilateral ovariectomy; OVX+ACEI(1)=bilateral ovariectomy and treatment with an ACE inhibitor, imidapril (1 mg kg<sup>-1</sup> per day); OVX+ACEI(3)=bilateral ovariectomy and treatment with an ACE inhibitor, imidapril (3 mg kg<sup>-1</sup> per day). \**P* < 0.05 vs. sham, †*P* < 0.05 vs. OVX, *N* = 6–10 per group. Bar indicates 0.3 mm.

**Table 1** Blood pressure and heart rate of each group

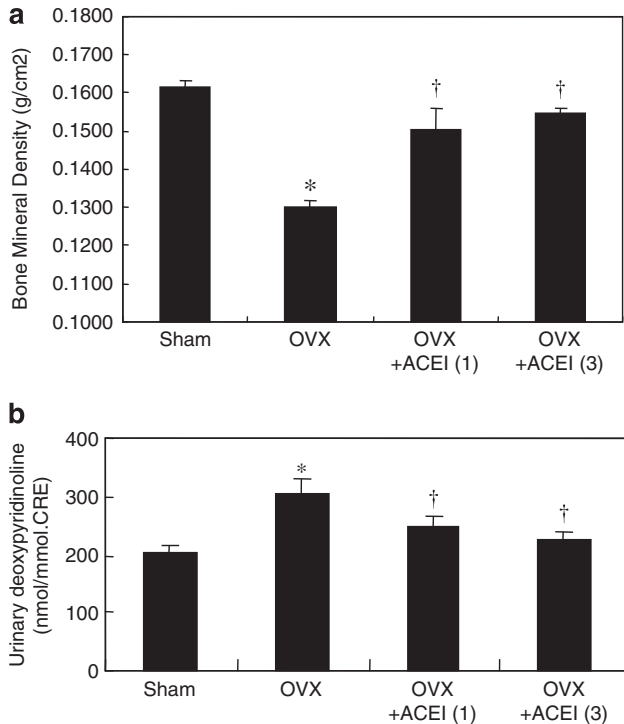
	SBP	DBP	HR	BW
Sham	148 ± 2.4	105 ± 3.4	350 ± 11.4	228 ± 4.5
OVX	146 ± 5.1	101 ± 3.4	358 ± 14.4	262 ± 3.2 <sup>#</sup>
+ACEI(1)	121 ± 1.5*	90 ± 2.7*	355 ± 7.2	256 ± 5.0
+ACEI(3)	119 ± 5.5*	84 ± 2.3*	323 ± 4.4	241 ± 4.8

Abbreviations: ACEI(1 or 3), angiotensin converting enzyme inhibitor, imidapril, (1 or 3 mg kg<sup>-1</sup> per day); BW, body weight (g); DBP, diastolic blood pressure (mm Hg); HR, heart rate (beats per minute, b.p.m.); OVX, ovariectomy; SBP, systolic blood pressure (mm Hg).  
\**P*<0.05 vs. OVX; <sup>#</sup>*P*<0.05 vs. sham.

**Table 2** Serum bone-related marker of each group

	Ca	P	ALP3	Osteocalcin
Sham	9.9 ± 0.3	6.3 ± 0.3	166 ± 6.0	951 ± 13.6
OVX	9.7 ± 0.3	6.4 ± 0.3	208 ± 8.7 <sup>#</sup>	1017 ± 19.9
+ACEI(1)	9.5 ± 0.3	6.2 ± 0.3	167 ± 3.6*	1016 ± 11.5
+ACEI(3)	9.5 ± 0.2	6.5 ± 0.2	172 ± 3.6*	1021 ± 12.4

Abbreviations: ACEI(1 or 3), angiotensin converting enzyme inhibitor, imidapril, (1 or 3 mg kg<sup>-1</sup> per day); ALP3, alkaline phosphatase isozyme 3 (IU l<sup>-1</sup>); Ca, calcium (mg dl<sup>-1</sup>); Osteocalcin (Gla-OC, ng ml<sup>-1</sup>); OVX, ovariectomy; P, phosphate (mg per 100 ml).  
<sup>#</sup>*P*<0.05 vs. sham. \**P*<0.05 vs. OVX.



**Figure 2** Effects of angiotensin-converting enzyme (ACE) inhibitor, imidapril, on urinary deoxyypyridinoline and bone density in ovariectomized spontaneous hypertensive rats (SHRs). (a) Bone mineral density by dual-energy X-ray absorptiometry (DEXA) and (b) urinary deoxyypyridinoline after 28 days of each treatment. Sham, sham operation; OVX, bilateral ovariectomy; OVX+ACEI(1), bilateral ovariectomy and treatment with an ACE inhibitor, imidapril (1 mg kg<sup>-1</sup> per day); OVX+ACEI(3)=bilateral ovariectomy and treatment with an ACE inhibitor, imidapril (3 mg kg<sup>-1</sup> per day). \**P*<0.05 vs. sham, <sup>†</sup>*P*<0.05 vs. OVX, *N*, 6–10 per group.

decreased in the tibia of ovariectomized SHR as compared with that in sham-operated rats (Figure 1c). In this model, the administration of imidapril (1 or 3 mg kg<sup>-1</sup> per day in drinking water) significantly decreased blood pressure, but not body weight, as shown in Table 1. The OVX-induced decrease in the ratio of ALP to TRAP activity was significantly ameliorated by imidapril (Figure 1c). Indeed, the TRAP-positive stained area was increased in the tibia of ovariectomized SHR, whereas treatment with imidapril significantly decreased the TRAP-positive stained area (Figure 1d). Importantly, these results were accompanied by a significant decrease in BMD in ovariectomized SHR, as assessed by DEXA, which was attenuated by the administration of imidapril (Figure 2a). Consistently, the increase in urinary deoxyypyridinoline was significantly attenuated by imidapril (Figure 2b). We further analyzed the serum bone-related markers in

this model. There is no difference in the serum calcium and phosphate level in each group, whereas the increase in the serum ALP3 level by OVX was significantly attenuated by imidapril (Table 2). The serum osteocalcin level and urinary calcium (data not shown) are not well correlated with the results in this model. These results suggest that an ACE inhibitor attenuated osteoporosis induced by estrogen deficiency and high blood pressure.

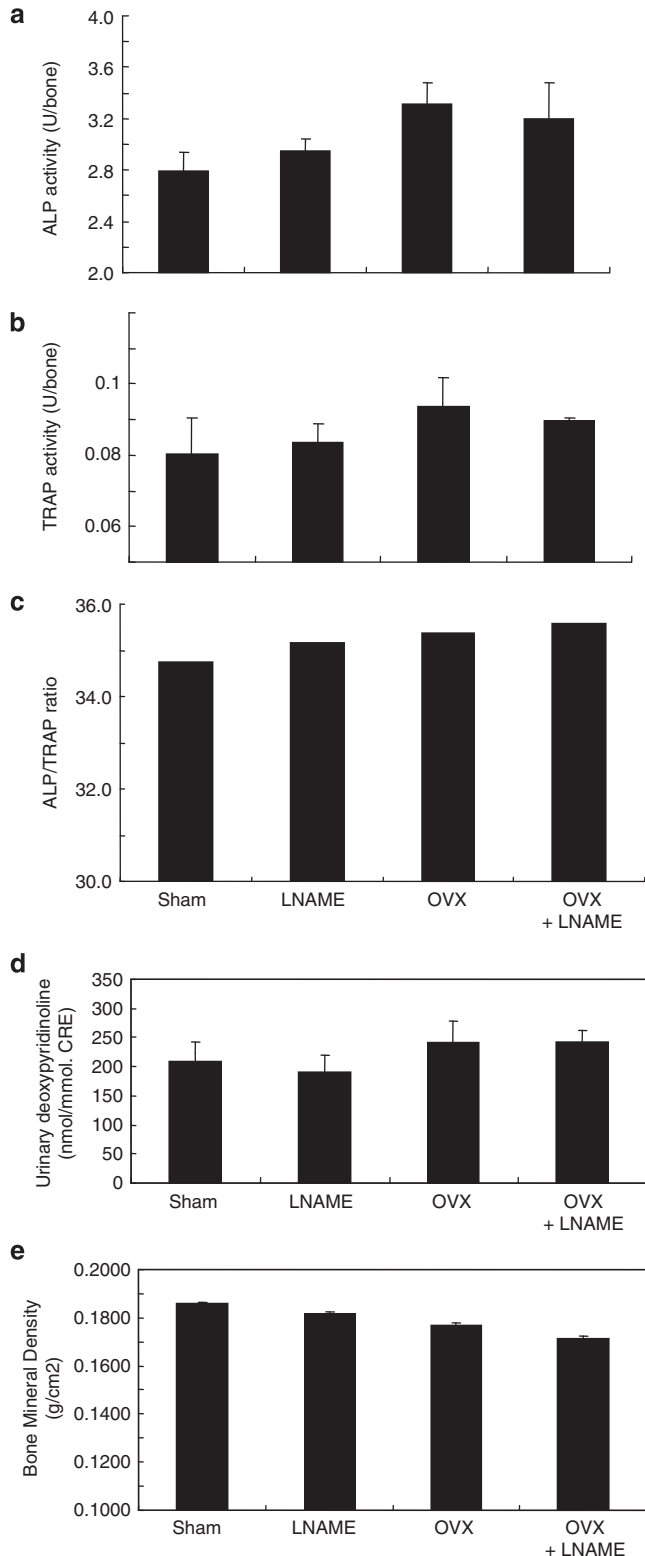
#### *In vivo* effects of L-NAME on osteoporosis in a rat OVX model

As ACE inhibitors possess the effects of both a blockade of RAS and an activation of the bradykinin–nitric oxide pathway, we examined the contribution of both pathways in an OVX-induced osteoporosis model. We have already reported that the administration of a suppressor dose of Ang II accelerated the increase in TRAP activity in the tibia, accompanied by a significant decrease in bone density and an increase in urinary deoxyypyridinoline. To clarify the contribution of the bradykinin–nitric oxide pathway to osteoporosis, we used a Wistar rat OVX model of estrogen deficiency with or without a long-term administration of L-NAME (1 mg ml<sup>-1</sup> in drinking water).<sup>16</sup> Unexpectedly, OVX did not induce osteoporosis in the Wistar rat model, and administration of L-NAME did not alter TRAP activity (Figures 3a–c), urinary deoxyypyridinoline (Figure 3d) or bone density (Figure 3e) in ovariectomized rats. We further examined the effect of imidapril (1 mg kg<sup>-1</sup> per day) with or without the administration of L-NAME in ovariectomized SHR. Similarly, the administration of L-NAME did not change ALP, TRAP and BMD (Figures 4a–d). These results suggest that activation of RAS, but not that of the bradykinin–nitric oxide pathway, might be involved in the process of estrogen deficiency-induced osteoporosis.

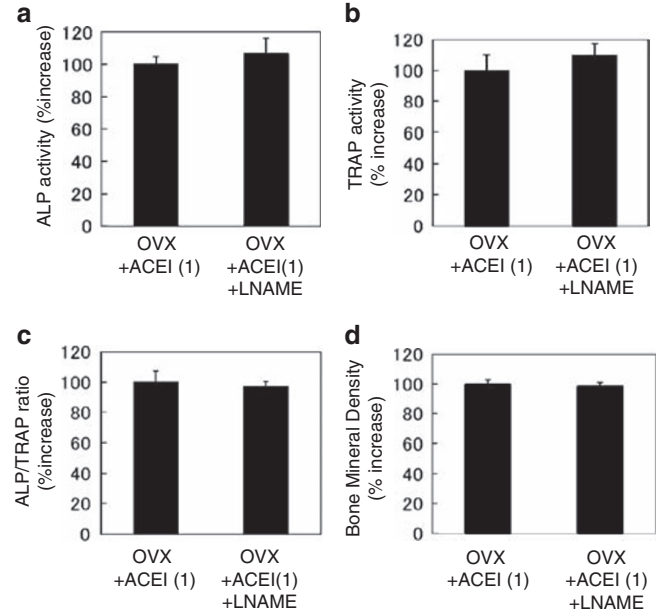
## DISCUSSION

Clinical epidemiological evidence has shown that high blood pressure is associated with an increase in bone loss, especially in elderly women.<sup>9</sup> Interestingly, an analysis of a retrospective case–control study in a large population of men and women with ages ranging from 30 to 79 years (601 fracture cases and 120 819 controls) has shown that thiazide diuretics, β-blockers and ACE inhibitors significantly reduced fracture risk. However, calcium channel blockers had no effect on fracture risk, and there is no evidence of a therapeutic efficacy of calcium channel blockers for the treatment of osteoporosis associated with hypertension. Interestingly, ACE inhibitors were effective in increasing BMD in women with the ACE DD polymorphism, which is associated with increased serum Ang II.<sup>19</sup>

Ang II indirectly promoted the differentiation and activation of osteoclasts responsible for bone resorption through the upregulation of the activator of the NF-κB (nuclear factor-κB) ligand (RANKL, also known OPGL, TRANCE and ODF) in osteoblasts.<sup>15,20</sup> Ang II may also influence calcium metabolism by decreasing ionized calcium and increasing the parathyroid hormone level.<sup>21</sup> Indeed, Ang II receptor



**Figure 3** Effects of nitro-L-arginine methylester (L-NAME) treatment in ovariectomized Wistar rats. (a) Alkaline phosphatase (ALP) activity, (b) tartrate-resistant acid phosphatase (TRAP) activity, (c) ratio of TRAP to ALP activity, (d) Urinary deoxypyridinoline and (e) bone mineral density determined by dual-energy X-ray absorptiometry (DEXA) after 28 days of each treatment. Sham, sham operation; L-NAME, administration of L-NAME (1 mg ml<sup>-1</sup> in drinking water); OVX, bilateral ovariectomy; OVX+L-NAME, bilateral ovariectomy and administration of L-NAME (1 mg ml<sup>-1</sup> in drinking water). *N*, 6–10 per group.



**Figure 4** Effects of angiotensin-converting enzyme (ACE) inhibitor, imidapril, with or without nitro-L-arginine methylester (L-NAME) treatment in ovariectomized spontaneous hypertensive rats (SHRs). (a) Alkaline phosphatase (ALP) activity, (b) tartrate-resistant acid phosphatase (TRAP) activity, (c) ratio of tartrate-resistant acid phosphatase (TRAP) to ALP activity, (d) bone mineral density determined by dual-energy X-ray absorptiometry (DEXA) after 28 days of each treatment. OVX+ACEI(1), bilateral ovariectomy and treatment with an ACE inhibitor, imidapril (1 mg kg<sup>-1</sup> per day); L-NAME, administration of L-NAME (1 mg ml<sup>-1</sup> in drinking water). Values are shown in percentage increase to OVX+ACEI(1), *N*, 6 per group.

antagonists (ARB) blocked osteoclast activation, leading to an attenuation of osteoporosis.<sup>15</sup> In addition, ACE inhibition also prevented osteoporosis, as shown in this study. However, ACE inhibitors also regulate the bradykinin–nitric oxide (NO) pathway as well as block Ang II production, different from ARB. It is well known that the NO pathway regulates local blood flow in bone marrow capillaries, which might enhance bone marrow formation. Thus, the contribution of the NO pathway might have a role in the prevention of osteoporosis by ACE inhibition.

Nevertheless, this study showed that a long-term administration of L-NAME did not accelerate osteoporosis, whereas the administration of a suppressor dose of Ang II accelerated the increase in TRAP activity in the tibia, accompanied by a significant decrease in bone density and an increase in urinary deoxypyridinoline.<sup>15</sup> Thus, the major contribution of ACE inhibitors toward the prevention of osteoporosis might be a result of the blockade of RAS rather than the activation of the bradykinin–NO pathway.

Previous animal studies showed that the administration of an ACE inhibitor, enalapril, did not cause significant changes in bone density, mineral content or morphometric parameters of the femur in 14-week-old ovariectomized female Wistar rats.<sup>22</sup> The difference from our study results might be because of the use of a different model (normotension vs. SHR). Similarly, in our study, OVX did not increase TRAP activity, urinary deoxypyridinoline or bone density in 10-week-old female Wistar rats. We speculate that high blood pressure is an important risk factor for osteoporosis through the activation of RAS. Interestingly, Imaoka *et al.*<sup>7</sup> previously demonstrated that the exaggerated natriuresis associated with an excessive loss of calcium in urine

may be involved in the abnormality of calcium metabolism in low-renin hypertensive elderly patients, but not in high-renin hypertensive patients. This suggests that the activation of RAS may be independent of the abnormality of calcium metabolism.

In vasculature, Horiuchi and colleagues<sup>23,24</sup> showed that estrogen antagonized Ang II signaling and Ang II induced atherosclerosis, which suggest that OVX might accelerate Ang II-induced signaling. Of importance, recent reports suggest that estrogen may enhance the ACE inhibition-mediated improvement of vascular remodeling in hypertension in female SHR with OVX through the inhibition of extracellular signal-regulated kinase 1/2.<sup>25</sup> It supports the impact of a cross talk between estrogen and Ang II signaling in female SHR.

Overall, an ACE inhibitor, imidapril, attenuated osteoporosis in hypertensive rats through the blockade of RAS. Owing to the increasing number of elderly people and the increase in the prevalence of osteoporosis, the need for focused preventive strategies has become a public health priority. On the basis of the findings presented in this study, together with clinical findings, ACE inhibitors as well as ARB might aid in the prevention of osteoporosis in hypertensive patients.

#### ACKNOWLEDGEMENTS

This work was partially supported by the National Institute of Biomedical Innovation, a Grant-in-Aid from the Ministry of Public Health and Welfare, a Grant-in-Aid from Japan Promotion of Science, and through Special Coordination Funds from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government. We thank Ms Natsuki Yasumasa for her technical support.

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