Int J Clin Exp Med 2015;8(8):12584-12592
www.ijcem.com /ISSN:1940-5901/IJCEM0011951

Original Article

Captopril, an angiotensin-converting enzyme inhibitor, possesses chondroprotective efficacy in a rat model of osteoarthritis through suppression local renin-angiotensin system

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Received June 26, 2015; Accepted August 11, 2015; Epub August 15, 2015; Published August 30, 2015

Abstract: Objective: A local tissue-specific renin-angiotensin system (local RAS) has emerged as a regulator of cartilage development and homeostasis. However, no report has described the chondroprotective efficacy of RAS inhibitor. Therefore, we studied the pharmacological function of captopril on hypertrophic differentiation of chondrocytes, cartilaginous degeneration and RAS components expression in a rat model of osteoarthritis (OA). Methods: OA was surgically induced in the right knee of male rats. Animal groups included age matched sham control (sham group), OA placebo (OA group), and OA treated with captopril (CAP group). Eight weeks after the induction of OA, the tibias were isolated and the sagittal sections were stained with Safranin O and Masson-Trichrome. The mRNA and protein expression of RAS components were measured by qRT-PCR and western blotting respectively. Results: The thickness of articular cartilage was reduced in the proximal tibia of the OA group, and decreased thickness of articular cartilage of the OA mice was effectively reversed by captopril treatment. Histological analyses revealed remarkable chondrocytes abnormality in OA rats, which were characterized by a marked expansion of hypertrophic zone and inhibition of proliferative zone of chondrocytes in the epiphyseal growth plate of tibia. However, captopril-treated could reverse chondrocytes abnormality in OA rats. Furthermore, the mRNA and protein expression of RAS components, renin, ACE, Ang II AT1R were upregulated in the proximal tibia of OA rats, however, the AT2R expression was suppressed. Intriguingly, captopril-treated could inhibit the activation of RAS in OA rats. Conclusions: The present study demonstrated that captopril could attenuate OA-induced osteoarticular injury, at least partially, through suppression local RAS.

Keywords: Osteoarthritis, chondrocytes, renin-angiotensin system, captopril

Introduction

Osteoarthritis (OA) is the most common form of arthritis and a widely prevalent disease characterized by the progressive destruction of extracellular matrix (ECM), articular cartilage and formation of osteophytes [1]. Although multiple factors are involved in triggering OA, the cartilage destruction is compromised at first, which appears to be a result of imbalance between ECM synthesis and degradation. During the development of OA, chondrocytes become metabolically active and disrupt the equilibrium between anabolic and catabolic effects [2]. In the mouse model or rabbit of osteoarthritis, Safranin O staining indicates degradation of articular cartilage on the tibial and femoral surfaces in the knee joint, moreover, increased subchondral bone formation and marginal osteophyte development are part of the joint pathology in OA [3, 4]. The ideal treatment of OA should focus on prevention of articular cartilage damage and many compounds are under investigation for this purpose [5]. A common practice includes administration of non-steroidal anti-inflammatory medicines, as well as injection of cortisone and hyaluronic acid [6]. Recent studies focused on addressing the ability of chondrocytes to repair cartilage in OA, for example, by increasing matrix synthesis in this avascular and alymphatic tissue [7]. Furthermore, surgical operation may be an ultimate choice for OA

treatment. However, it introduces the risk of infection and damage to surrounding structures.

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 [8]. Recent studies indicate that the components of RAS, such as renin, angiotensin-converting enzyme (ACE), and angiotensin II (Ang II) receptors, are expressed in the local milieu of bone [9, 10]. Clinical studies show that ACE and renin are upregulated in synovial stroma in rheumatoid arthritis (RA) [11, 12]. In Asian populations, ACE gene polymorphism is found to be associated with primary knee OA [13, 14]. Chondrocytes from all patient types express angiotensin II (Ang II) type 1 receptor (AT1R) and AT2R mRNA in OA or RA patients [15]. In C57BL/6 adult mice, hypertrophic chondrocytes of epiphyseal plates included in the tibia and the lamina terminals express local RAS components, and activation of AT1R suppressed and activation of AT2R enhanced the expression of markers of hypertrophic differentiation [16, 17]. Intriguingly, continuous infusion of Ang II modulates hypertrophic differentiation and apoptosis of chondrocytes in cartilage formation in a fracture model mouse [18]. These studies mainly attempted to establish a link between these RAS components and OA. Moreover, we think RAS is important to investigate cartilage hypertrophy and diseases induced by hypertrophic changes like osteoarthritis. However, the chondroprotective efficacy of RAS inhibitors and the underlying molecular mechanisms regulating chondrogenesis during osteoarthritis development are still poorly understood.

We recently performed an animal study to address the effects of the captopril on articular cartilage of Sprague-Dawley rats with osteoarthritis. The aim of the present study was to identify the pathophysiological role of the local RAS in articular cartilage, and above all, to elucidate the impact of the captopril on the cartilaginous degeneration of osteoarthritic rat.

Materials and methods

Animal treatment

Six-month-old male Sprague-Dawley rats (Slac Laboratory Animal, Shanghai, China) were allowed to acclimate to the environment for 1 week. All experimental procedures were carried out in accordance with the guidelines of the Shanghai Third People's Hospital, Shanghai JiaoTong University on Animal Care. The right knee joint was exposed with a medial par patellar approach. The patella was dislocated laterally and the knee placed in full flexion, followed by anterior cruciate ligament transection and medial meniscus resection with micro-scissors. Sham-arthrotomized animals were negative controls. The rats were randomly divided into three groups: (1) Sham group (n = 12); (2) OA group (n = 12); (3) OA with captopril-treated group received captopril orally at a dose of 10 mg/kg per day (CAP, n = 12). All rats were sacrificed 8 weeks after captopril treatment.

Bone histomorphology

The tibias were decalcified in 0.5 M EDTA (pH = 8.0) and then embedded in paraffin by standard histological procedures. Section of 5 μm were cut and stained with hematoxylin & eosin (H&E) and Masson-Trichrome staining and visualized under a microscope (Leica DM 2500).

Tissue sections were graded using the scoring system described by Glasson et al [19].

Because of the specific procedure taken for OA induction, we focused on the histological evaluation of the articular cartilage. Accordingly, the grade was assigned by two independent scorers blinded to treatment allocation (KH, AN) as follows: "0" = normal; "0.5" = loss of Safranin O without structural changes; "1" = small fibrillations without loss of cartilage; "2" = vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina; and "3" to "6" = vertical clefts/erosion to the calcified cartilage extending to < 25%, 25% to 50%, 50% to 75%, and > 75% of the articular surface, respectively.

Quantitative real-time PCR

The RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNAs was performed by reverse transcription reactions with 2 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Promega, Switzerland) with oligo dT (15) primers (Fermentas) as described by the manufac-

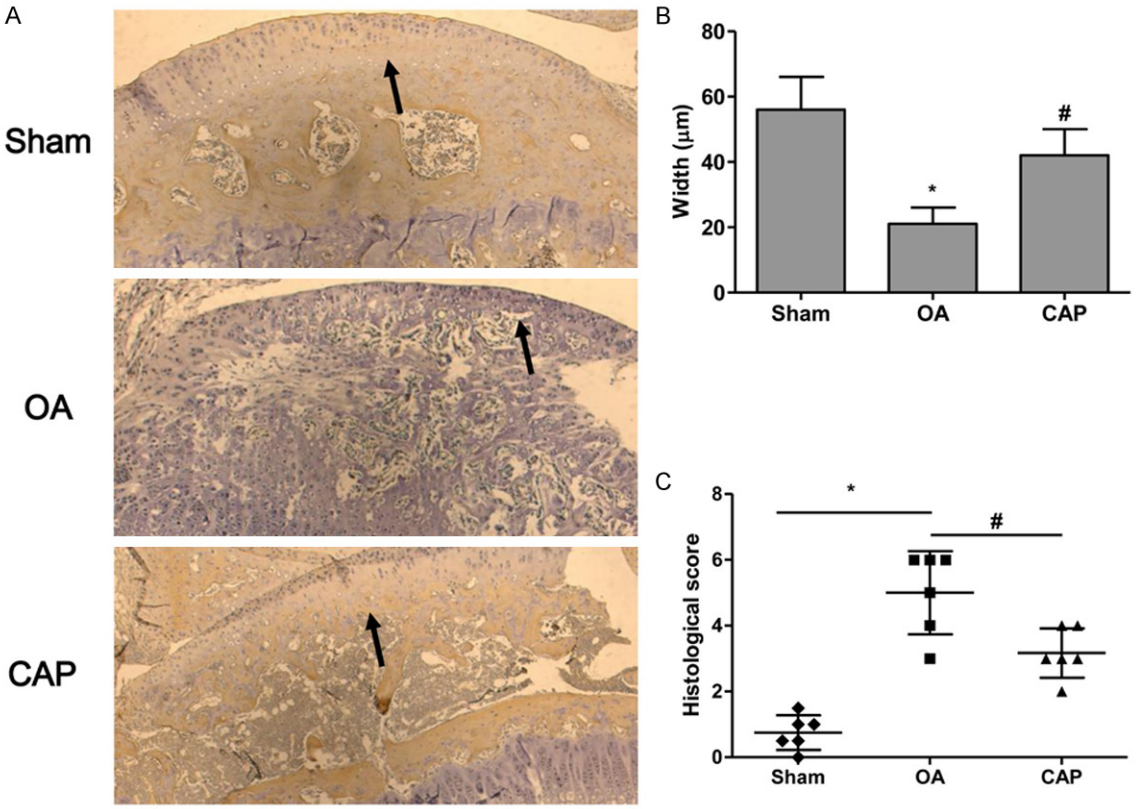

Figure 1. Images of safranin O staining of the sagittal knee joint section. Samples eight weeks after induction of osteoarthritis, the tibias were stained by safranin O staining (A). The thickness of articular cartilage is shown by black arrows (magnification, × 50) and the width of the articular cartilage was quantified (B). Graphs showed histological scores of tibias for progression of osteoarthritis (C). Values are expressed as mean ± SEM, n = 6 in each group. $^{*}P < 0.05$, versus Sham group; $^{\#}P < 0.05$, versus OA group.

turer. The first strand cDNAs served as the template for the regular polymerase chain reaction (PCR) performed using a DNA Engine (ABI 9700). The cycling conditions were 2-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 55°C for 60 s. Glyceraldehyde-phosphate dehydrogenase (GAPDH) as an internal control was used to normalize the data to determine the relative expression of the target genes. The reaction conditions were set according to the kit instructions. After completion of the reaction, the amplification curve and melting curve were analyzed. Gene expression values are represented using the $2^{-\Delta\Delta Ct}$ method. PCR with the following primers: renin, Forward 5'-GAGGCCTTCCTTGACCAATC-3' and Reverse 5'-TGTGAATCCCACAAGCAAGG-3'; renin-receptor, Forward 5'-CTCCCAGCGAGGAGAGAGTGTAT-3' and Reverse 5'-ATGTAGCACTTGCAGTTCGGAGAGA-3'; AGT, Forward 5'-CGAGTGGGAGAGGTTCTCAA-3' and Reverse 5'-CTCGTAGATGCGAACAGGA-3'; ACE, Forward 5'-CCCATCTGCTAGGGAACATGT-3' and Reverse 5'-GGTGTCCATCCCTGCTTTATCA-3'; AT1R, Forward 5'-TGCTCACGTGTCTCAGCATC-3' and Reverse 5'-TTTGGCCACCAGCATCGTG-3'; AT2R, Forward 5'-TAAGCTGATTTATGATAACTGC-3' and Reverse 5'-ATATTGAACTGCAGCAACTC-3'; GAPDH, Forward 5'-ATGGTGAAGGTCGGTGTGA-3' and Reverse 5'-CCATGTAGTTGAGGTCAATGAG-3'.

Western blotting

The proximal tibias were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 60 μg of protein were separated on 10% SDS-PAGE gel, transferred to PVDF Transfer Membrane (Millipore). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with the following antibodies, renin, ACE, Ang II, AI1R and AT2R

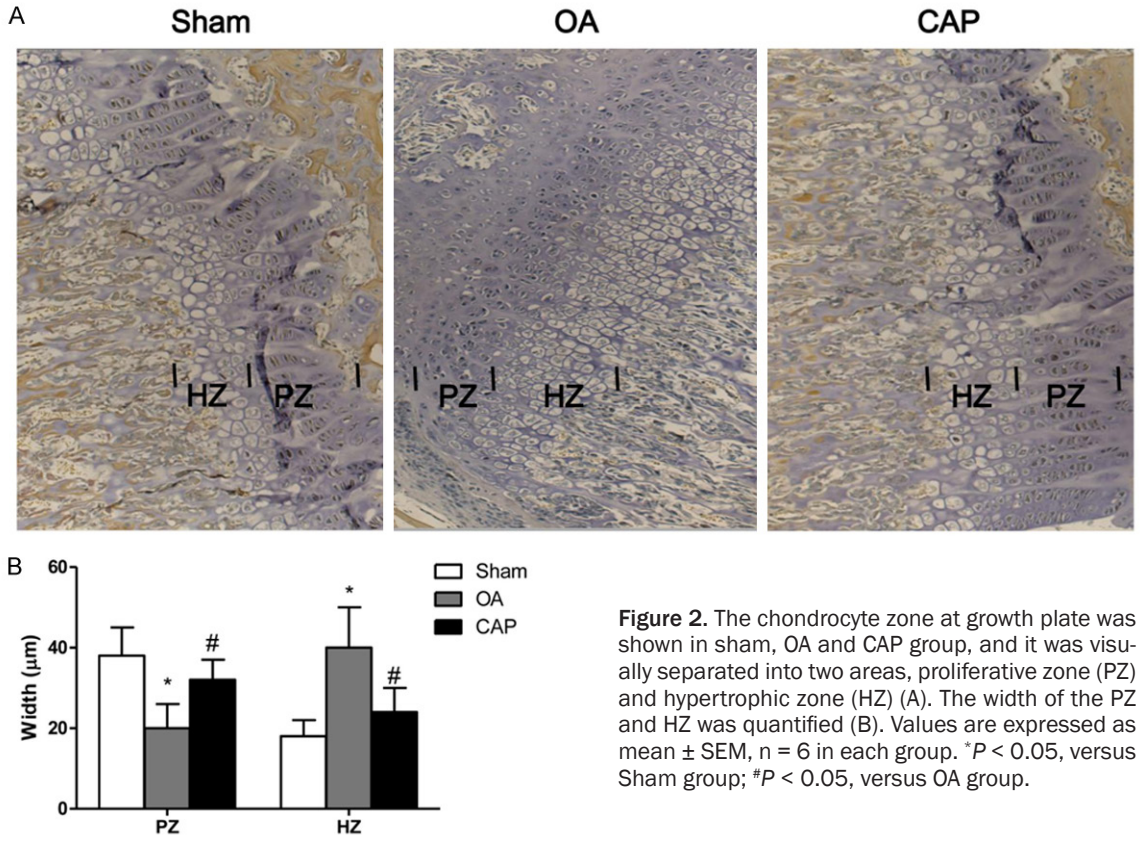

Figure 2. The chondrocyte zone at growth plate was shown in sham, OA and CAP group, and it was visually separated into two areas, proliferative zone (PZ) and hypertrophic zone (HZ) (A). The width of the PZ and HZ was quantified (B). Values are expressed as mean ± SEM, n = 6 in each group. $^{*}P < 0.05$, versus Sham group; $^{\#}P < 0.05$, versus OA group.

(Santa Cruz, USA), at dilutions ranging from 1:500 to 1:2,000 at 4°C over-night. After three washes with TBST, membranes were incubated with secondary immunoglobulins (Igs) conjugated to IRDye 800 CW Infrared Dye (LI-COR), including donkey anti-goat IgG and donkey anti-mouse IgG at a dilution of 1:10,000-1:20,000. After 1 hour incubation at 37°C, membranes were washed three times with TBST. Blots were visualized by the Odyssey Infrared Imaging System (LI-COR Biotechnology). Signals were densitometrically assessed (Odyssey Application Software version 3.0) and normalized to the β-actin signals to correct for unequal loading using the monoclonal anti-β-actin antibody (Bioworld Technology, USA).

Statistical analysis

The data from these experiments were reported as mean ± standard errors of mean (SEM) for each group. All statistical analyses were performed by 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 $P < 0.05$. Differences with P value of < 0.05 were considered statistically significant.

Results

Reduced cartilage degradation and expansion of hypertrophic zone of chondrocytes by captopril

In the rat model of osteoarthritis, the Safranin O staining indicated that the degradation of articular cartilage on the tibial surfaces (black arrows) in the knee joint (**Figure 1A**). The intensity of Safranin O staining decreased in the samples harvested eight weeks after the induction of OA. However, the Safranin O staining was partially restored by daily administration of captopril at a dose of 10 mg/kg in OA rats (**Figure 1A**). The thickness of articular cartilage was reduced in the proximal tibia of the OA group (shown by arrow) suggesting the degradation of articular cartilage in the knee joint. The decreased thickness of articular cartilage of the OA mice was effectively reversed by captopril treatment (**Figure 1B**). The histological

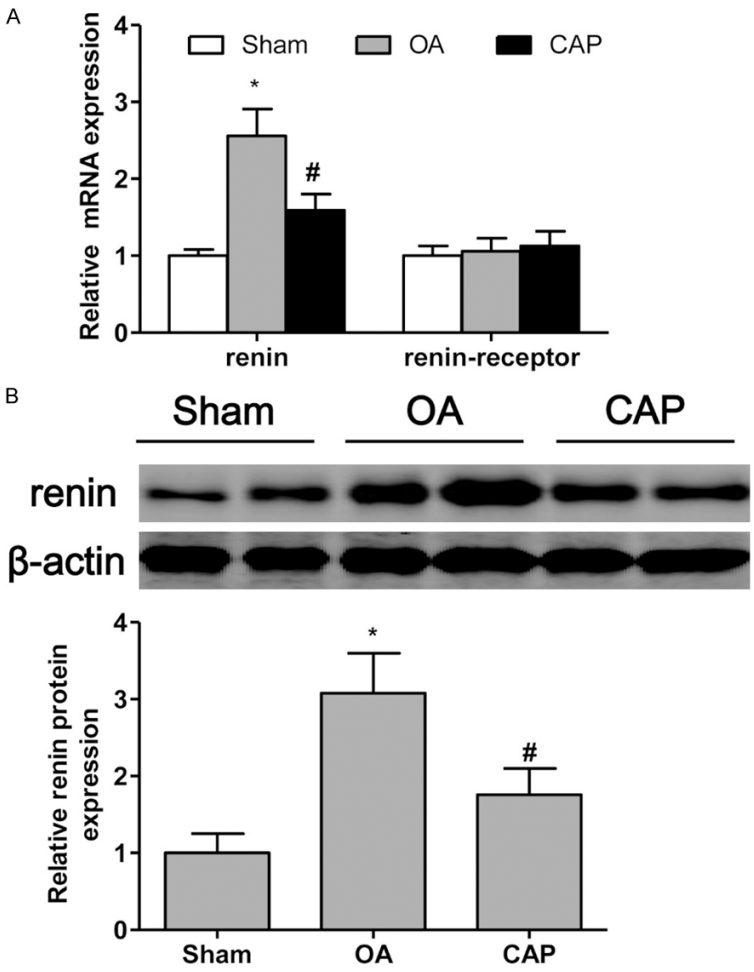

Figure 3. The expression of renin and renin-receptor in the proximal tibias. The mRNA expression of renin and renin-receptor was measured by qRT-PCR (A). The protein expression of renin was measured by western blotting (B). Values are expressed as mean ± SEM, n = 6 in each group. $^{*}P < 0.05$, versus Sham group; $^{\#}P < 0.05$, versus OA group.

score (0 for normal, and 6 for worst OA) revealed that captopril significantly reduced OA-linked tissue degeneration. In OA group, the histological mean scores for tibial plateau were 0.75 (0.5) in sham group, 5 (1.3) in OA group and 3.2 (0.75) in CAP group (**Figure 1C**).

Masson staining of the proximal epiphyseal growth plate of the tibia from rat revealed the process of chondrocyte differentiation in OA (**Figure 2**). Histological analyses revealed remarkable chondrocytes abnormality in OA rats. These were characterized by a marked expansion of hypertrophic zone and inhibition of proliferative zone of chondrocytes in the epiphyseal growth plate of tibia (**Figure 2A** and **2B**). However, captopril-treated could reverse chondrocytes abnormality in OA rats.

Captopril inhibits local RAS activity in the proximal tibia of OA rats

We first examined the mRNA and protein expression levels of renin in the proximal tibia of OA rats using qRT-PCR analysis and western blotting respectively. The results showed that the mRNA and protein expression of renin were significantly increased in proximal tibia of OA rats compared to those of sham rats, and further down-regulated in the captopril-treated group (**Figure 3A** and **3B**). However, the mRNA expression of renin-receptor had no significant difference among three experimental groups. Previous study demonstrated that Ang II promoted hypertrophic differentiation of chondrocytes and reduced apoptosis of hypertrophic chondrocytes independently of high blood pressure. In the present study, we found that the mRNA and protein expression of ACE were significantly higher than that of the sham group. Still, captopril could inhibt the expression of ACE in the proximal tibia of OA rats (**Figure 4A** and **4B**). Intriguingly, captopril could also downregulate the expression of AGT (**Figure 4C**), which is the precursor of Ang II that is formed from angiotensin I by ACE, a key bioactivator in RAS. Furthermore, we indicated that the protein expression of Ang II was increased in the proximal tibia of OA rats compared to those of sham rats, however, captopril-treated could suppress Ang II expression in the proximal tibia of OA rats (**Figure 4D**). The receptors expression of Ang II were examined by qRT-PCR and western blotting. The results showed that the mRNA (**Figure 5A**) and protein (**Figure 5B**) expression of AT1R were increased in OA group as compared to that of sham group, as well as AT2R mRNA and protein expression were decreased in the proxi-

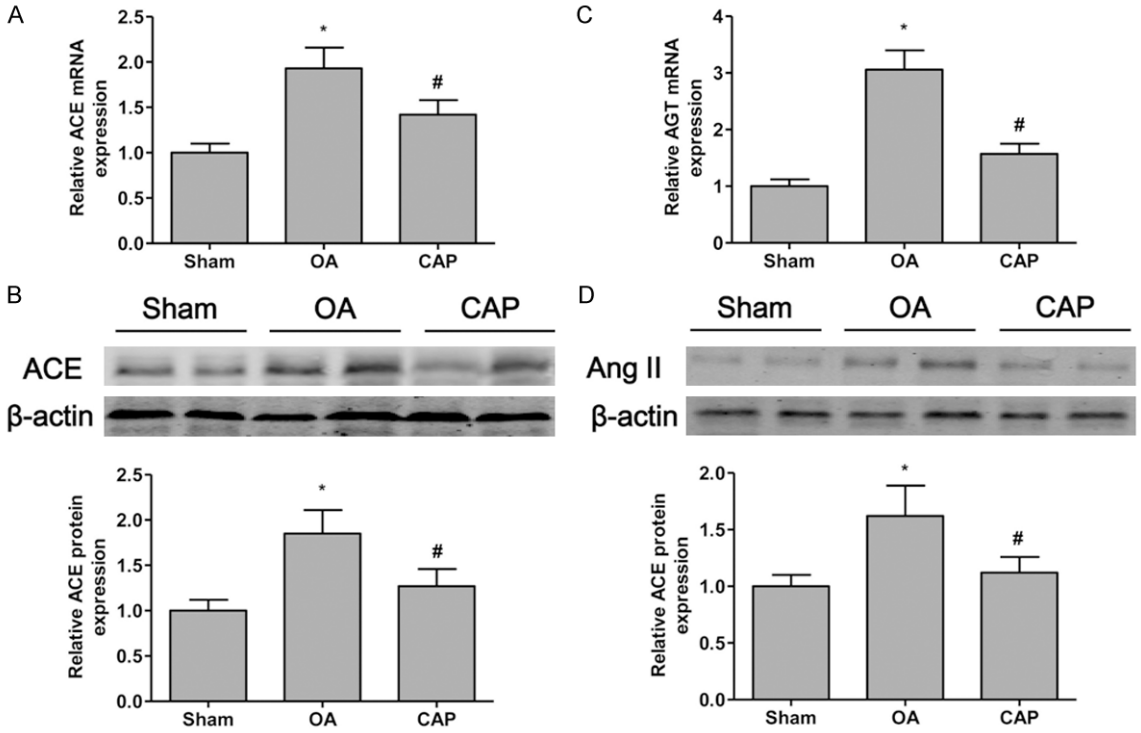

Figure 4. The expression of ACE and Ang II in the proximal tibias. The mRNA (A) and protein (B) expression of ACE were measured by qRT-PCR and western blotting respectively. The AGT mRNA (C) and Ang II protein (D) expression were measured by qRT-PCR and western blotting respectively. Values are expressed as mean ± SEM, n = 6 in each group. $^{*}P < 0.05$, versus Sham group; $^{\#}P < 0.05$, versus OA group.

mal tibia of OA rats (**Figure 5C** and **5D**). However, the increased expression of AT2R and decreased expression of AT1R in the proximal tibias of the captopril group was statistically significant compared to those of the OA group.

Discussion

A local tissue-specific RAS has been identified in many organs [20]. Mounting evidences have showed that RAS play a vital role in the regulation of bone metabolism, and Ang II accelerates osteoporosis by activating osteoclasts, and treatment with ACE inhibitors is associated with a reduced fracture risk [9, 21]. Emerging datas strongly implicate RAS components in chondrocytes differentiation and osteoarticular diseases, such as osteoarthritis and rheumatoid arthritis [12, 15-17]. For example, activation of AT2R enhanced the expression of markers of hypertrophic differentiation in ATDC5 cell lines [17]. Moreover, Ang II induces hypertrophic differentiation and apoptosis of chondrocytes, however, olmesartan can reverse the increase in apoptotic cells and the decrease in anti-apoptotic genes induced by Ang II infusion [18]. Based on these studies, we could know clearly that the activation of local RAS was correlated with chondrocyte dysfunction, which was an essential factor on triggering cartilaginous degeneration and OA. Therefore, we proposed the hypothesis that captopril, a RAS inhibitor, had a function to attenuate articular cartilage injury in OA through suppression local RAS.

Here we revealed that RAS components, such renin, AT1R, ACE and Ang II, were significantly increased, as well as AT2R was decreased in the proximal tibia of OA rats, however, the renin-receptor was no obvious difference. Moreover, the findings of our study revealed that captopril exerted chondroprotection and inhibited cartilaginous degeneration in a rat model of OA. Intriguingly, the expression of renin, ACE and Ang II was significantly lower in the captopril-treated OA rats compared with that of OA rats. In contrast, expression of AT2 receptors was

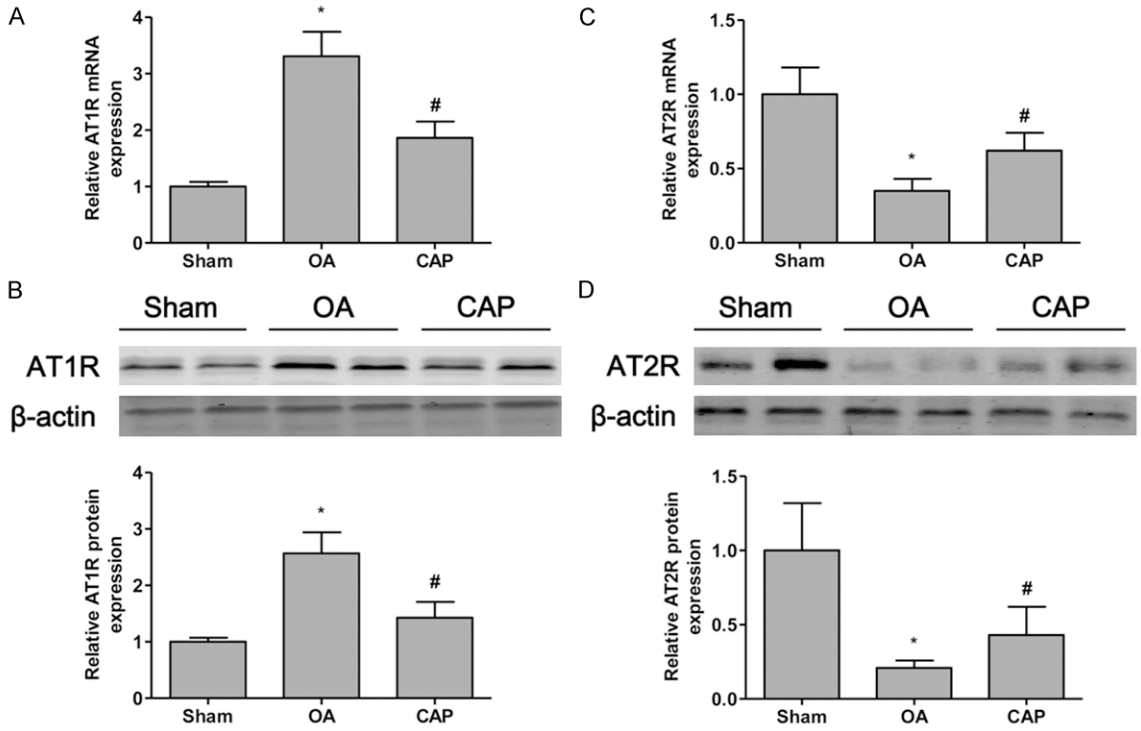

Figure 5. The expression of AT1R and AT2R in the proximal tibias. The mRNA (A) and protein (B) expression of AT1R were measured by qRT-PCR and western blotting respectively. The mRNA (C) and protein (D) expression of AT2R were measured by qRT-PCR and western blotting respectively. Values are expressed as mean ± SEM, n = 6 in each group. $^{*}P < 0.05$, versus Sham group; $^{\#}P < 0.05$, versus OA group.

significantly greater in captopril-treated OA rats, whereas decreased in expression of AT1 receptors were observed. These results suggested that Ang II signalling via its receptors, AT1 and AT2, plays a key role in pathological alterations of articular cartilage in OA rats. However, the counter-expression of AT1R and AT2R brought to our attention. In a murine femur fracture model, AT1R and AT2R are detected in the periosteal callus, and perindopril-treated stimulates fracture healing and periosteal callus formation, at at least partially, through upregulation AT2R and downregulation ACE [22]. Moreover, osteoblasts and hypertrophic chondrocytes express ACE during endochondral bone formation in the periosteal callus area and are localized in the zone of cartilage maturation and hypertrophy [4, 7, 22]. In our study, we found that the expression of ACE was increased, and a marked expansion of hypertrophic zone and inhibition of proliferative zone of chondrocytes were confirmed in the epiphyseal growth plate of tibia. Interestingly, captopril-treated could simultaneously suppress OA-induced ACE expression and hypertrophic chondrocytes. We also found that significant suppression of cartilage degradation was identified eight weeks after the induction of OA in the tibia.

Our experimental study is the first to report the chondroprotective efficacy of captopril in a rat model of osteoarthritis. The hypertrophic chondrocytes and cartilage degradation were alleviated by captopril, and the tissues-local RAS was suppressed by captopril treatment. Collectively, the present study demonstrated that captopril could attenuate OA-induced osteoarticular injury, at least partially, through suppression local RAS. However, the progression of symptoms of OA differs between the tibia and femur, and the efficacy of drugs depends on time points [3]. The chondrocytes dysfunction and the pharmacological roles of captopril in the femur of OA rats still need to be further investigated.

Disclosure of conflict of interest

None.

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