Role of the local bone renin-angiotensin system in steroid-induced osteonecrosis in rabbits

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Received August 5, 2013; Accepted February 11, 2014

DOI: 10.3892/mmr.2014.1978

Abstract. The specific pathogenesis of steroid-induced osteonecrosis (ON) is yet to be elucidated and until recently effective prophylactic therapies have not been available. The local renin-angiotensin system (RAS) exists in the bone and has an important role in local bone regulation. However, to the best of our knowledge, the interrelation between local bone RAS and steroid-induced ON is yet to be investigated. In the present study, 45 rabbits were injected with a single intramuscular dose of 20 mg/kg methylprednisolone acetate (MPA) and were sacrificed 1 (group A), 2 (group B) and 3 (group C) weeks subsequent to MPA administration (n=15 per group). Ten rabbits were used as a control group (group N). The presence or absence of ON in the bilateral femoral heads was examined histopathologically. The mRNA and protein expression of components of the RAS, including angiotensin II (Ang II), angiotensin converting enzyme (ACE) and Ang II type 1 (AT1) and Ang II type 2 (AT2) receptors, were detected in the bone. Significant changes in Ang II, ACE, and AT1 and AT2 receptor expression were observed in the bone of the rabbits in the different groups. Moreover, the expression of Ang II and ACE was highest one week subsequent to administration of the glucocorticoid methylprednisolone and the expression of the AT1 and AT2 receptors was highest two weeks following methylprednisolone administration. ON occurs most significantly at three weeks following the administration of MPA in this animal model, thus the changes in Ang II, ACE and AT1 and AT2 receptor expression preceded this. The present study found that ON was strongly associated with the activation of the local bone RAS in rabbits.

Introduction

Osteonecrosis (ON) is one of the most serious complications induced by high doses and/or long-term administration of glucocorticoids (GCs). Several mechanisms have been associated with the pathogenesis of ON, including intraosseous hypertension, oxidation injury, apoptosis, hypercoagulability and lipid metabolism disorders (1-5). However, the precise mechanism underlying the pathogenesis of steroid-induced ON is yet to be elucidated and until recently, effective prophylactic therapies have not been available.

The renin-angiotensin system (RAS) is classically known to be a circulating endocrine system regulating blood pressure and electrolyte homeostasis. The main effector peptide in RAS is angiotensin (Ang) II, which is formed from Ang I by angiotensin-converting enzyme (ACE). Ang II exerts its biological effects through binding to specific angiotensin receptors, primarily the Ang II type 1 (AT1) receptor. In addition to this classical systemic RAS, additional local tissue-specific RASs have been identified in various organs and tissues, including the heart, kidney, bone marrow, blood vessels and fat tissues. Moreover, the local RAS has been identified to have an important role in local organ regulation (6).

The local RAS has been shown to exist in bone tissue (7,8) and its activation in bone tissue has been found to induce metabolic bone disorders (9-11). Furthermore, Ang II-induced signaling in vascular and endothelial cells promotes reactive oxygen species (ROS) production, platelet activation, inflammation and altered vasoreactivity, all of which impair bone microcirculation (12).

Previous studies have shown that GCs stimulate ACE expression in bovine aorta endothelial cells, rat cardiac fibroblasts and vascular smooth muscle cells (13-15). Furthermore, Sato et al (16) showed that GCs upregulate the expression of the AT1 receptor in vascular smooth muscle cells. However, little is known about the role of GCs in the regulation of the RAS in the bone.

In the present study, it was hypothesized that GCs may activate the local bone RAS and that this activation may be involved in the pathogenesis of steroid-induced ON. Therefore,
this study investigated the effect of steroid-induced ON on the expression of Ang II, ACE and AT₁ and Ang II type 2 (AT₂) receptors in adult female Japanese white rabbits.

Materials and methods

Animals. The experimental protocol was approved by the institutional animal use and care review board of Xi'an Jiaotong University (Xi'an, China). Fifty-five adult, female Japanese white rabbits (weight, 3.3-4.2 kg; age, 30-32 weeks; Animal Center of Xi'an Jiaotong University) were investigated. All rabbits were housed at the Animal Center of Xi'an Jiaotong University and maintained on a standard diet and water.

Forty-five rabbits were injected once with 20 mg/kg body weight methylprednisolone acetate (MPA; Pfizer, Inc., Brussels, Belgium) into the right gluteal muscle, and were then divided into three groups (A, B and C) consisting of 15 rabbits per group. The rabbits in groups A, B and C were sacrificed by overdose of anesthesia at 1, 2 and 3 weeks subsequent to MPA administration, respectively. The control group (group N) consisted of 10 rabbits, which were maintained under the same conditions as the treatment groups, but were not injected with MPA (17). Immediately following sacrifice, one half of each femoral head was isolated and fixed in 10% neutral buffered formalin, decalcified using 13% EDTA and embedded in paraffin. The other half of each femoral head was frozen and stored at -80°C for additional examinations.

Assessment of ON. One 4-µm thick section of each femoral head was cut in the coronal plane and stained with hematoxylin and eosin. The presence or absence of ON was determined in whole areas of two sections for each rabbit. The sections were examined using light microscopy (Nikon YS100; Nikon Corporation, Toyko, Japan) by two blinded pathologists. ON was identified based on the presence of empty lacunae or pyknotic osteocyte nuclei in the bone trabeculae, as well as the presence of necrosis in the surrounding bone marrow or fat cells. Empty lacunae in the bone trabeculae, but without bone marrow or fat cell necrosis was not classified as ON (Fig. 1).

Rabbits were considered to have ON based on the identification of ON in at least one of the two sections analyzed. The incidence of ON was calculated as the ratio of the number of rabbits with ON to the total number of rabbits (17,18).

Immunohistochemistry. Immunohistochemistry was performed using one 4-µm thick section of each femoral head in order to assess the presence of AT₁ receptors and ACE using specific antibodies according to the manufacturer's instructions. Briefly, subsequent to deparaffinization, sections were treated with 3% hydrogen peroxide for 20 min to inhibit endogenous peroxidase activity. Antigen retrieval was then performed using 0.01 M citrate buffer (pH 6.0) at 80°C for 10 min. Sections were preincubated with normal goat serum (Biosynthesis Biotechnology Co. Ltd., Beijing, China) for 30 min at room temperature, prior to incubation at 4°C overnight with mouse anti-rabbit ACE (ab11734; Abcam PLC, Cambridge, MA, USA) and AT₁ receptor (ab9391; Abcam PLC) monoclonal antibodies, diluted 1:20 and 1:50 in phosphate-buffered saline, respectively. Sections were then incubated with secondary goat anti-mouse antibodies (Biosynthesis Biotechnology Co. Ltd.) and with horseradish peroxidase (HRP)-labeled streptavidin (Biosynthesis Biotechnology Co. Ltd.). The final reaction product was visualized using diaminobenzidine. Images were captured using the QWin550CW Image Acquiring and Analysis system (Leica Microsystems, Wetzlar, Germany). Heart tissue was used as a positive control and showed positive brown staining. Sections without primary antibody-treatment were used as negative controls.

The intensity of AT₁ receptor and ACE immunostaining in groups N, A, B and C were quantitatively analyzed using the analysis software Image-Pro Plus (Media Cybernetics, Baltimore, MD, USA). One section was obtained from each rabbit and 10 images were captured from each section, which were analyzed for positive staining at a magnification of x400. The total area of each analyzed section was the same. Integrated optical density (IOD) was assessed, in which ‘integrated’ refers to the sum of all the pixel intensity or density values in a given image. The IOD values obtained from the 10 images in each section were averaged and compared with the averaged IOD values of each section.

Western blot analysis. Six femoral heads were selected randomly from each group for western blot analysis. Total protein was isolated by homogenizing the femoral head using radioimmunoprecipitation assay buffer (RIPA) buffer. The concentration of total protein was quantified using the bicinchoninic acid (BCA) protein assay reagent (Pierce™, Rockford, IL, USA). Laemmli buffer (5X) was added to each sample to a final concentration of 1X, and 20 µl of each preparation was loaded onto 5 and 10% SDS polyacrylamide gels. SDS-PAGE was performed using a constant voltage of 90 V for 100 min. Following electrophoresis, proteins were transferred onto 0.45 µm nitrocellulose and polyvinylidene fluoride membranes (Hybond-ECL; Amersham Pharmacia Biotechnology Inc., Piscataway, NJ, USA) and blocked with 3% bovine serum albumin at room temperature for 2 h. Membranes were incubated overnight at 4°C with anti-AT₁ receptor and -ACE primary antibodies (Abcam PLC) diluted 1:400 and 1:100, respectively. Membranes were then incubated with HRP-labeled goat anti-mouse secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunoreactive proteins were visualized on a film using an enhanced chemiluminescence kit (NEN Life Science Products Inc., Boston, MA, USA). Relative protein expression was determined using image analysis software (Media Cybernetics). β-actin was detected using a mouse monoclonal anti-actin antibody (1:3,000; Santa Cruz Biotechnology, Inc.) and was used as an internal control.

Analysis of ACE activity in the serum and bone. Prior to sacrifice, blood was collected from all rabbits without anticoagulant and was stored on ice. Blood was then centrifuged at 1,848 x g for 10 min. The serum was obtained and stored at -80°C until required for the ACE assay. A total of 200 mg bone tissue was obtained from each rabbit and homogenized in ice-cold Tris-HCl buffer solution (1 ml/100 mg sample wet weight). The buffer solution consisted of 20 mM Tris-HCl (pH 8.3), 5 mM Mg(CH₃COO)₂, 30 mM KCl, 250 mM sucrose and 0.5% Nonidet P-40. The homogenized samples were centrifuged for 30 min at 11,300 x g at 4°C. The protein
concentration in the supernatant was quantified using the BCA protein assay reagent (Pierce). The supernatant was stored at -80°C until required for the ACE assay.

ACE activity in the serum and the supernatant was determined by analyzing the production rate of hippuric acid from the synthetic tripeptide substrate hippuryl-L-histidyl-L-leucine (HHL) as described previously (19). The serum and the supernatant were incubated with the substrate, HHL, and the hippuric acid concentration was assessed using ultra violet absorbance at 228 nm. ACE activity was expressed as nmol/min/mg protein or pg/ml serum. All analyses were performed in duplicate.

**Analysis of Ang II concentration in the plasma and bone.** Prior to sacrifice, blood was collected from all rabbits with a mixture of protease inhibitors (0.30 M EDTA, 0.32 M dimer-captopril dimercaptopropanol and 0.34 M 8-sulphydryl quinoline sulfate) and stored on ice. Blood was then centrifuged at 943 x g for 7 min. The plasma was obtained and stored at -80°C until required for the Ang II assay. A total of 100 mg bone tissue was obtained from each rabbit and homogenized and extracted in lysis buffer containing 10 mM Tris, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton X-100, 0.02% NaN₃ and 0.2 mM phenylmethylsulfonyl fluoride protease inhibitor cocktail. The homogenized samples were then centrifuged for 30 min at 11,300 x g at 4°C. The protein concentration in the supernatant was quantified using the BCA protein assay reagent (Pierce). The supernatant was stored at -80°C until required for the Ang II assay. The concentration of Ang II was measured using radioimmunoassay (RIA) (20) with a commercial RIA kit (Beifang, Tianjin, China). The concentration of Ang II was expressed as pg/mg protein or pg/ml plasma. All analyses were performed in duplicate.

**Quantitative polymerase chain reaction (qPCR) analysis.** Total RNA was isolated by homogenizing the femoral heads using the TRIzol® protocol. cDNA was synthesized using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. Samples were analyzed using SYBR-Green® PCR Master mix (DRR820S; Takara Bio, Inc., Shiga, Japan) and an ABI 7300 Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The sequences of the primers used for qPCR were as follows: Forward: 5'-TGTAGCCAAAGTCACCTGCATC-3' and reverse: 5'-ACTCGTAATGGAAACAAACC-3' for the AT₁ receptor; forward: 5'-ATAAGCCCATCAATAAGCAG TTAG-3' and reverse: 5'-GAGGAAGGTAGCCACAAGG-3' for the AT₂ receptor; forward: 5'-GGAGCATACCAAAGTAAGCAG ATGAAAG-3' for ACE; and forward: 5'-GGCCCGGGAACCATC AAGGAGA-3' and reverse: 5'-AGAAGGGAGGGG TCAGGAGAG-3' for β-actin. Relative mRNA expression was quantified using the 2-ΔΔCt method, in which ΔΔCt=(Ctgene-Ctβ) / (Ctcontrol-Ctβ). The relative quantities of the AT₁ and AT₂ receptors and ACE were normalized to the quantity of the β-actin transcript in the same sample. All assays were performed in triplicate.

**Statistical analysis.** All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The incidence of ON was compared using the χ² test or Fisher's exact test. All data are expressed as the mean ± standard deviation and compared using one way analysis of variance or the Kruskal-Wallis test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Incidence of steroid-induced ON.** Five of the 55 rabbits died following MPA injection and were excluded from the experiments. One was in group A, two were in group B and two were in group C. The remaining rabbits were alive until the end of the experiment. ON was not found in group N, whereas three of the 14 rabbits in group A, six of the 13 rabbits in group B and 10 of the 13 rabbits in group C were observed to develop ON. The incidence of ON in group C (77%) was significantly higher than that in group A (21%; P=0.007), but not significantly different from that in group B (46%; P=0.236). Compared with group A, the incidence of ON in group B was found to increase, but there was no significant difference between the two groups (P=0.236) (Fig. 1).

**AT₁ receptor and ACE protein expression in bone.** Immunohistological analysis of the femoral heads showed that the AT₁ receptor and ACE were expressed in the osteoblasts, osteoclasts and bone marrow cells of the bone tissue, but were not expressed in osteocytes (Fig. 2). This finding is consistent with previous studies (8,21,22). Immunostaining for AT₁ receptor was observed to increase in group A and was most significant in group B, compared with group N (Fig. 2). Quantitative image analysis of AT₁ receptor immunostaining revealed an increase in group A compared with group N (P=0.037), and an increase in group B compared with groups N and C (P=0.004 and P=0.032, respectively) (Fig. 3). Immunostaining for ACE was highest in group A (Fig. 2). Quantitative image analysis of immunostaining for ACE demonstrated a significant increase in group A compared with groups N and C (P=0.002 and P=0.037, respectively), and a significant increase in group B compared with group N (P=0.026) (Fig. 3).

Western blot analysis revealed a significant increase in AT₁ receptor expression in group A compared with group N (P=0.004) and in group B compared with groups N and C (P<0.001). Furthermore, the protein expression of ACE was observed to increase in group A compared with groups N, B and C (P=0.001, P=0.023 and P=0.001, respectively), and significantly increased in groups B and C compared with group N (P=0.002 and P=0.041, respectively) (Fig. 4).

**Ang II concentration and ACE activity.** The concentration of Ang II in the bone was found to increase in groups A, B and C compared with group N (P=0.001, P=0.023 and P=0.078, respectively). The concentration of Ang II in the bone was observed to decline among groups A, B and C; however, these changes were not significant (Table I). The activity of ACE in the bone of rabbits in group A was higher than that in groups N, B and C (P<0.001, P=0.002 and P<0.001, respectively). Furthermore, the activity of ACE in groups B and C was found to increase compared with group N (P<0.001). The concentration of Ang II in the plasma and the activity
Figure 1. Histological features of ON in rabbits. (A and B) Normal bone tissue harvested from group N showing no ON. (C and D) Normal bone tissue harvested from group A showing empty lacunae containing no osteocytes (arrow) and no bone marrow or fat cell necrosis. (E and F) Osteonecrotic bone tissue harvested from group C. Necrotic trabeculae show pyknosis and empty lacunae (arrow) with marked degeneration of the surrounding bone marrow haematopoietic cells and adipocytes. Stain, hematoxylin and eosin. Magnification, (a, c and e) x100; (b, d and f) x400. ON, osteonecrosis.

Figure 2. Immunohistochemical staining of the AT1 receptor and ACE in rabbit femoral heads from groups N, A, B and C. AT1 receptor and ACE are expressed in osteoblasts (blue arrows), osteoclasts (red arrows) and bone marrow cells (black arrows), but are not expressed in osteocytes (white arrows). Magnification, x400. AT1, Ang II type 1; ACE, angiotensin converting enzyme.
of ACE in the serum were not observed to differ significantly among the groups (Table I). These changes in local and systemic RAS activity are similar to those observed by Mitani et al (19), who found that cholesterol increased local tissue ACE activity but not serum ACE activity in rabbits, suggesting that the systemic RAS may be more stable than the local RAS.

mRNA expression of AT1 and AT2 receptors and ACE in the bone. The mRNA expression of AT1 and AT2 receptors and ACE in groups A, B and C was significantly higher than that in group N (P<0.05). The mRNA levels of the AT1 and AT2 receptors in group B were the highest of the 4 groups. Furthermore, the mRNA expression of ACE in group A was the highest of the 4 groups (Fig. 5).

Discussion

In the present study, ON was first observed one week subsequent to MPA injection and was most significant three weeks following injection. The incidence of ON was 77% three weeks following MPA administration, which was similar to that observed in the study by Iwakiri et al (17), in which the incidence of ON was 83% three weeks subsequent to steroid injection. In the present study, the expression of Ang II, ACE, and the AT1 and AT2 receptors significantly increased one week following MPA administration, concurrent with the onset of ON in this animal model. Moreover, the expression of Ang II and ACE was highest one week subsequent to steroid administration and the expression of the AT1 and AT2 receptors was highest two weeks following MPA administration. These changes in expression following MPA injection, precede the time at which ON occurs most significantly in this animal model, which is three weeks. This suggests that ON is strongly
The activation of the local RAS in the bone microcirculation may lead to vessel contraction, vascular inflammation, endothelial cell dysfunction and hypercoagulability of the bone microcirculation, which decrease the blood supply and ischemia of the femoral head. These alterations induce a pathological condition increasing the risk of ON.

The second possible mechanism underlying the pathogenesis of ON involves altered bone metabolism. In the local milieu of the bone, two major types of cells, osteoblasts and osteoclasts, coordinate resorb and form the bone matrix, which conserves the bone architecture and mass during adulthood (34). However, the balance between osteoblasts and osteoclasts is disturbed by the activation of the local bone RAS. Previous studies have shown that Ang II suppresses osteoblastic cell differentiation and bone formation, and decreases calcium uptake into the bone (9,10). In addition, Ang II activates osteoclasts and stimulates bone resorption (35). In a clinical study, patients treated with an ACE inhibitor exhibited increased bone mineral density and reduced fracture rate (11). Therefore, the activation of the local bone RAS may suppress bone formation and stimulate bone resorption, causing a bone metabolism disorder through altering the balance between bone formation and bone resorption, increasing the risk of ON development and impairing ON repair.

In summary, GCs may activate the local bone RAS, which may impair bone microcirculation and bone metabolism. These effects, in conjunction with numerous other factors, including alterations in lipid metabolism and intraosseous hypertension, may induce ON.

In the present study, the expression of Ang II, ACE, and the AT$_1$ and AT$_2$ receptors significantly increased one week following MPA administration, and the concentration of Ang II and the activity of ACE in the bone were highest one week subsequent to MPA injection. However, the expression of the AT$_1$ and AT$_2$ receptors was greatest two weeks following MPA injection potentially due to the overproduction of Ang II one week following MPA injection. Thus, Ang II may stimulate the expression of the AT$_1$ and AT$_2$ receptors, which has been reported in a previous study (36). Therefore, the administration of ACE inhibitor (ACEI) or angiotensin receptor blocker (ARB) in rabbits may inhibit the GC-induced activation of the local bone RAS, which may represent a preventive treatment for steroid-induced ON. This will be investigated in our future studies.

The present study has certain limitations. The data show that the expression of the components of the local bone RAS, including Ang II, ACE and the AT$_1$ and AT$_2$ receptors were enhanced by MPA in the femoral head, but the precise role of the local bone RAS in the development of ON requires further investigation. Thus, future studies will be performed in order to demonstrate the interrelation between the activation of local bone RAS and the development of ON, as well as the effect of ACEI or ARB on preventing steroid-induced ON.

In conclusion, activation of the local bone RAS may not be the sole cause of steroid-induced ON, and the precise role of the local bone RAS in the pathogenesis of ON requires further investigation. However, the present study has contributed to an enhanced understanding of the molecular processes underlying ON. Furthermore, these findings suggest the possibility

![Diagram](https://example.com/diagram.png)

Figure 6. Schematic representation of the potential mechanisms underlying ON. Glucocorticoids activate the local bone RAS, which induces bone microcirculation and bone metabolism disorders generating an environment that favours the development of ON. ON, osteonecrosis; RAS, renin-angiotensin system.

associated with changes in the expression of components of the RAS, including Ang II, ACE, and the AT$_1$ and AT$_2$ receptors.

Although the precise mechanism underlying the pathogenesis of steroid-induced ON is yet to be elucidated, studies of the pathophysiology of ON have shown that GCs induce ischemia of the femoral head and an imbalance in osteoblast and osteoclast activity, leading to ON, which is highly likely to underlie the pathogenesis of steroid-induced ON (23-25). Previous studies have shown that the activation of the local bone RAS induces metabolic bone disorders (7,9,26) and impairs bone microcirculation (27). In the present study, the GC MP was found to activate the local bone RAS, which is similar to the effect of GCs on the local RAS reported in other tissues (13,16). Therefore, steroid-induced ON may be closely associated with the local bone RAS in rabbits.

In the present study, two potential mechanisms were hypothesized to underlie the activation of the local bone RAS and induce ON following steroid administration (Fig. 6). The first possible mechanism involves a disruption in the microcirculation of bone. Ang II is one of the most potent microvascular vasoactive agents (28), and leads to vessel contraction and decreased blood supply of the femoral head. Ang II induces the production of adhesion molecules, chemokines and inflammatory cytokines, including vascular cell adhesion molecule-1, intercellular adhesion molecule-1, E-selectin, monocyte chemotactractant protein-1, interleukin-6, interleukin-8 and tumor necrosis factor-$

- \alpha$ in endothelial cells (29). These molecules induce endothelial cell dysfunction, abnormal blood coagulation and thrombi formation, which may lead to ischemia of the femoral head (30). Ang II upregulates NADPH oxidase components, thereby enhancing the production of ROS in the microcirculation of the bone (31). ROS are potent inter- and intracellular second messengers, which mediate vessel inflammation (32). Elevated ROS levels induce growth arrest and increase the rate of senescence and apoptosis in endothelial cells. Ang II also stimulates the expression of plasminogen activator inhibitor-1 (33), which alters homeostatic mechanisms that balance thrombosis with fibrinolysis and lead to hypercoagulability of the bone microcirculation.
of preventive approaches to steroid-induced ON through blocking the activation of the local bone RAS.

Acknowledgements

This study was partially supported by a grant from the National Natural Science Foundation of China (grant no. 81101337). The authors would like to thank the pathologists Mr. Xiaoge Zhao and Mr. Gang Cui from the Medical College of Xi'an Jiaotong University (China) for their contributions.

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