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Involvement of skeletal renin–angiotensin system and kallikrein–kinin system in bone deteriorations of type 1 diabetic mice with estrogen deficiency

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ABSTRACT

Aims: This study was aimed to investigate the involvement of skeletal renin–angiotensin system (RAS) and kallikrein–kinin system (KKS) in bone deteriorations of mice in response to the combination treatment of estrogen deficiency and hyperglycemia.
Methods: The female C57BL/6J mice were sham-operated or ovariectomized with vehicle or streptozotocin (STZ) treatment. Two weeks later, the biochemistries in serum and urine were determined by standard colorimetric methods or ELISA. The H&E and TRAP staining were performed at the tibial proximal metaphysis. The polymerase chain reaction and immunoblotting were applied for molecular analysis on mRNA and protein expression.
Results: The mice after treating with ovariectomy and STZ showed the decreased level of serum Ca and the increased level of serum PTH and urine Ca. The H&E staining showed trabecular bone abnormalities as demonstrated by the loss, disconnection and separation of trabecular bone network as well as the loss of chondrocytes and appearance of chondrocyte cluster at growth plate of tibia. The significant increase of matured osteoclast number was shown in group with double treatments. The combination treatment significantly up-regulated mRNA expression of AGT, ACE, renin receptor, MMP-9 and CAII, and protein expression of renin, and decreased the ratio of OPG/RANKL and the expression of bradykinin receptors in bone tissue.
Conclusion: Ovariectomy combined with STZ induction produced more detrimental actions on bone through the activation of local bone RAS and the down-regulation of bradykinin receptors, as compared to the respective single treatment.

1. Introduction

It is now known that, in addition to the classical renin-angiotensin system (RAS) in circulation, there exists local RAS in many tissues, and that the active agent angiotensin II (Ang II) within RAS plays a major role in the initiation and progression of tissue diseases and some metabolic disorders (Rodriguez-Perez et al., 2010). As a key active peptide in RAS, Ang II could accelerate osteoporosis of ovariectomized (OVX) rats (Shimizu et al., 2008). Ang II has been shown to stimulate the production of the osteoclastogenic cytokine, receptor activator of NF-κB ligand (RANKL), by acting on osteoblasts, thereby indirectly increasing the differentiation of osteoclasts (Kaneko et al., 2011). Additionally, Ang II destroys osteoblastic function and inhibits bone formation by directly acting on osteoblasts via regulating target genes including Cbfa1, SOST, MMP-3 and MMP-13, and the relevant signaling pathways like MAPK and JNK (Guan, Zhou, & Li, 2011; Kaneko et al., 2011; Li et al., 2014; Paszty, Turner, & Robinson, 2010).

The mRNA expression of angiotensin-converting enzyme (ACE) and renin-receptor, and the protein expression of renin and Ang II were markedly up-regulated in bone tissue of type 1 diabetic male mice induced by streptozotocin (Diao et al., 2014; Zhang, Diao, et al., 2014). Actually, the emerging experimental evidence demonstrated the involvement of local bone RAS in the development of bone injuries in different animal models, such as aged mice (Gu, Zhang, Li, et al., 2012), chronic kidney disease-induced osteoporotic mice (Gu, Zhang, Wu, Diao, Gebru, & Deng, 2012) and glucocorticoid-induced osteoporotic rabbits (Zhang, Wang, et al., 2014). The blockade of Ang II signaling by Ang II type 1 receptor or the reduction of Ang II

Conflict of interest: The authors declare that they have no conflict of interest.

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production by ACE inhibitor could effectively improve bone disorders (Donmez et al., 2012; Garcia et al., 2010; Shimizu et al., 2008), suggesting the biological roles of local RAS in maintaining bone health.

Menopause is a risk factor for osteoporosis as the estrogen deficiency is known to impair bone function and metabolism (Sajjan et al., 2012). The clinical and animal studies indicated an inverse association between the circulating estrogen level and the activation of local RAS (Rodriguez-Perez et al., 2010; Yung et al., 2011). Several lines of evidence suggested estrogen favorably modulates the RAS (O'Donnell, Floras, & Harvey, 2014; Zhang et al., 2016). Conversely, estrogen deficiency due to menopause may contribute to over activity of the tissue RAS and the replacement of estrogen therapy could reverse these changes in OVX rats (Dean, Tan, O'Brien, & Leenen, 2005; Yung et al., 2011). Type 1 diabetes is occasionally accompanied with postmenopausal osteoporosis (Nicodemus & Folsom, 2001; Schwartz et al., 2001). It was reported that hyperglycemia could lead to bone deteriorations of male mice due to the increased activity of skeletal RAS as described by our previous studies (Diao et al., 2014, 2013; Zhang, Diao, et al., 2014).

Additionally, bradykinin, major component of kallikrein-kinin system (KKS), regulates osteoblast differentiation and osteoclast maturation (Wu, Ai, Chen, Zhao, & Liu, 2016), consequently managing bone metabolism through modulating osteogenesis and osteoclastogenesis. However, whether the skeletal RAS and KKS are locally involved in the bone deteriorations caused by estrogen deficiency combined with hyperglycemia is not known yet.

Therefore, the aim of the present work was to investigate the involvement of the skeletal RAS and KKS in bone deteriorations induced by a combination of hyperglycemia and estrogen deficiency using OVX mice, an animal model widely employed to mimic postmenopausal metabolism syndrome, and injected with streptozotocin inducing type 1 diabetes.

2. Materials and methods

2.1. Animal treatment

Ten-week-old female ICR mice (Slac Laboratory Animal, Shanghai, China) were allowed to acclimate to the environment for 1 week. The mice were randomly divided into four groups: (1) sham operated control mice (C, n = 6); (2) ovariectomized (OVX) mice (O, n = 6); (3) streptozotocin (STZ)-induced hyperglycemia mice (S, n = 6); (4) OVX mice with STZ treatment (OS, n = 6). The animals were rendered hyperglycemia one week after ovariectomy surgery by intraperitoneal injection of STZ, dissolved in citrate buffer (0.1 M at pH 4.2), at 40 mg/kg body weight for 5 consecutive days. The mice in C group and O group were treated with vehicle only. All mice, with pair feeding and free access to water, were housed at 22 °C with a 12-h light/dark cycle in metal cages. The mice were sacrificed 2 weeks after STZ injection. The fasting blood glucose (FBG) level was measured with a blood glucose monitoring system (Roche). The body weight was recorded before collecting samples. The animal study protocol was reviewed and approved by the institution's Animal Ethics Committee at Shanghai University of Traditional Chinese Medicine.

2.2. Chemistries in serum and urine

The concentrations of calcium (Ca) and creatinine (Cr) from 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 intact parathyroid hormone (PTH 1–84) were detected using mouse bioactive PTH ELISA assay (Immutopics, Inc., San Clemente, CA, USA).

2.3. Bone histology

The tibias were decalcified in 0.5 M EDTA (pH = 8.0) and then embedded in paraffin by standard histological procedures. Sections of 3 μm were cut and stained with hematoxylin & eosin (H&E). Trabecular bone mass expressed as trabecular bone area over total area (BA/TA) was measured using an OsteoMeasure system (OsteoMetrics Inc., Decatur, GA, USA). Tartrate-resistant acid phosphatase (TRAP) staining was used for the identification of osteoclasts following the manufacturer's instructions (Sigma, St Louis, MO, USA). The numbers of osteoclast were quantified 1–4 mm from growth plates in bone marrow cavity. The assays were scored by averaging the number of osteoclasts per low-power field in at least three fields per slide. Stained slides were visualized under microscope (Leica DM 2500).

2.4. Reverse transcription–polymerase chain reaction

The tibia of each animal was crushed under liquid nitrogen conditions and RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, 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) 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). β-2 M 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 (Gu, Zhang, Li, et al., 2012 Zhang, Dong, Leung, & Wong, 2009).

2.5. Western blotting

The femurs 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 primary antibodies, including mouse anti-renin monoclonal antibody, goat anti-bradykinin receptor-1 (B1R) polyclonal antibody, and goat anti-B2R polyclonal antibody (Santa Cruz Biotechnology, USA). After three washes with TBST, the membrane was incubated with donkey anti-mouse IgG conjugated to IRDye 800CW Infrared Dye (LI-COR). 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 (Bioworld Technology, USA).

2.6. Statistical analysis

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

3. Results

3.1. Basic parameters and biomarkers in serum and urine

The body weight did not change among the groups (Table 1). Two weeks after the STZ injection, the fasting blood glucose (FBG) value in