Adrenomedullin inhibits osteoclast differentiation through the suppression of receptor activator of nuclear factor-κB ligand-induced nuclear factor-κB activation in glucocorticoid-induced osteoporosis

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Received September 7, 2016; Accepted April 21, 2017

DOI: 10.3892/etm.2017.5025

Abstract. The current study aimed to improve the understanding on the association between adrenomedullin and osteoporosis in mice with glucocorticoid-induced osteoporosis. Bone resorption and osteoporosis-associated indexes, including maximum load, stiffness, energy to failure, ultimate strength, elastic modulus, post-yield displacement and post-yield displacement, in mice with osteoporosis were analyzed in order to evaluate the effect of adrenomedullin. The receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation was investigated subsequent to treatment with adrenomedullin *in vitro*. The results demonstrated that adrenomedullin significantly improved bone mass loss, density, bone strength and osteoporosis disease in the mice with glucocorticoid-induced osteoporosis. In addition, adrenomedullin markedly improved the osteoporosis-associated NFATc1, TRAP, OSCAR and c-Fos expression levels. Furthermore, the current findings indicated that RANKL-mediated osteoclast differentiation was suppressed *in vitro* and *in vivo*. Notably, the data revealed that adrenomedullin significantly improved the osteoporotic symptoms through inhibition of RANKL-induced NF-κB activation in glucocorticoid-induced osteoporosis. In conclusion, adrenomedullin serves an essential role in the progression of glucocorticoid-induced osteoporosis, regulating the bone mass loss, density and strength through the NF-κB signaling pathway.

Introduction

Bone tissue is a dynamic structure that presents continuous remodeling and metabolism coupled with the action of osteoblasts and osteoclasts (1). The functions of osteoblasts and osteoclasts serve an important role in maintaining the normal skeletal system function (2,3). Dysfunction of osteoblasts and osteoclasts frequently leads to bone disease (4). Osteoclasts are responsible for bone resorption caused by bone microstructure damage and bone-associated disorders (5). Currently, statistical data have indicated that osteoporosis is becoming a serious health problem in the aging population since it results in weakened bone structure and fractures in the elderly (6,7). Pathological research has indicated that osteoclast activity and subtypes are crucial for the treatment of osteoporosis (8). The clinical consequences of osteoporosis include fractures of the upper extremities, hips and even spine, resulting in the loss of function and independence of patients, impairing their quality of life, as well as increasing morbidity and mortality (9). Therefore, it is important to further understand the molecular mechanism of osteoporosis and develop more efficient drugs for the treatment of this disease. The present study investigated the molecular mechanism of glucocorticoid-induced osteoporosis in osteoblasts and osteoclasts in mice.

Adrenomedullin is a 52-amino acid regulatory peptide that is coded by the gene location on mouse chromosome 7, which has a ubiquitous distribution and various physiological functions (10). In recent years, adrenomedullin has been reported to serve a multifunctional role in numerous human diseases due to its anti-inflammatory, anti-apoptotic, anti-oxidative and anti-cancer properties (11). In addition, Hu *et al* (12) have identified that plasma concentration levels of calcitonin gene-related peptide and adrenomedullin are associated with the disease progression in patients with primary osteoporosis. The plasma levels of adrenomedullin receptor may affect the function of adrenomedullin in the progression of osteoporosis. Furthermore, Kim *et al* (13) have demonstrated that adrenomedullin prevented bone loss in a mice model of postmenopausal osteoporosis. A previous study also showed that adrenomedullin was able to attenuate interleukin-1β-induced inflammation and apoptosis in rat Leydig cells through inhibition of nuclear factor-κB (NF-κB) signaling pathway (12). Notably, receptor activator of NF-κB ligand (RANKL)-induced osteoclast differentiation through the inhibition of cFos protein proteolysis has been investigated via inhibition

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Key words: osteoporosis, osteoclast differentiation, adrenomedullin, nuclear factor-κB

of IkB degradation (13,14). Antibodies that target tumour necrosis factor (TNF)-α and RANKL may ameliorate local inflammation and osteoporosis (15). Additionally, cyclooxygenase (COX) 2 induction is associated with osteoporosis by regulation the ratio of osteoblast and osteoclast (16). These previous studies have suggested that adrenomedullin shows a beneficial effect in the treatment of osteoporosis. However, the molecular mechanism of adrenomedullin for the glucocorticoid-induced osteoporosis has not been investigated to date.

In the present study, the therapeutic effects of adrenomedullin on glucocorticoid-induced osteoporosis were investigated. The bone mass loss, bone density, bone strength and osteoporosis disease were examined, and the mRNA expression levels of osteoporosis-associated factors, including NF of activated T-cells 1 (NFATc1), tartrate-resistant acid phosphatase (TRAP), osteoclast-associated immunoglobulin-like receptor (OSCAR) and c-Fos, in osteocytes from experiment mice were detected (17-19). Furthermore, the study analyzed the signaling pathway mediated by adrenomedullin in mice with osteoporosis and researched the efficacy of adrenomedullin treatment on RANKL-induced osteoclast differentiation. The observed data suggested that adrenomedullin inhibits osteoclast differentiation through suppression of RANKL-induced NF-κB activation in glucocorticoid-induced osteoporosis.

Materials and methods

Ethics statement. The present study was conducted in strict accordance with the Care and Use of Laboratory Animals guidelines of the Chinese PLA General Hospital (Beijing, China). All experimental protocols were approved by the Ethics Committee of the Chinese PLA General Hospital. All surgery and euthanasia procedures ensured minimization of suffering.

Animals. A total of 42 female ICR mice (age, 6-8 weeks old; weight, 33-38 g) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were feed under pathogen-free conditions and maintained at a controlled environment (temperature, 23±1˚C; humidity, 50-60%) with an artificial simulation of 12 h light/dark cycles. All mice received intravenous injection of glucocorticoid (Sigma Aldrich; Merk KGaA; Darmstadt, Germany; 5 mg/kg) once a day for 15 days in order to induce osteoporosis. The mandibular cortical width was used as an indicator to determine that the osteoporosis model was successfully established, as previously described (20). Because diphosphonate is an efficient drug for osteoporosis therapy, diphosphonate was used to as a positive control group (21). ICR mice with osteoporosis were randomly divided into three groups (n=14 per group) and received 14-day once daily intraperitoneal injection treatment as follows: Adrenomedullin (0.5 mg/kg), diphosphonate (0.5 mg/kg) or phosphate-buffered saline (PBS; serving as the untreated osteoporosis model group, 0.5 mg/kg). The drug treatment procedures were conducted according to a previous study (22). The therapeutic efficacies of adrenomedullin were analyzed assessing bone characteristics according to a previous study (23).

Bone resorption assays. Experimental mice were sacrificed on day 15 and long bones were obtained. To generate osteoclasts or osteoblasts, $5x10^4$ cells were plated per well in 24-well tissue culture plates and treated with different doses of M-CSF (5 mg/ml) or RANKL (5 mg/ml), respectively as described previously (24) Cells cultured in the presence of 10^{-6}M 1,25-dihydroxyvitamin D_3 and 10^{-6} M prostaglandin E_2 (Sigma-Aldrich; Merck, Darmstadt, Germany) for 7 days at 37˚C. The total bone resorption was measured using ImagePro Plus version 4.0 (Media Cybernetics, Inc., Silver Spring, MD, USA) as described previously (25).

Analysis of adrenomedullin effects on osteoclast differentiation. Bone marrow cells (BMCs) were obtained from adrenomedullin-, diphosphonate- and PBS-treated ICR mice with osteoporosis on day 15 as described previously (26) and cultured in minimum essential medium (MEM, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) at 37˚C. The suspending BMCs were carefully collected by centrifuging at 300 x g for 10 min at room temperature and cultured for 72 h in 10 ng/ml macrophage colony-stimulating factor (M-CSF) at 37˚C, followed by addition of adrenomedullin (10 ng/ml), diphosphonate (10 ng/ml) or the same volume of PBS. The TRAP activity was then measured using the TRAP kit (cat. no. 387A-1KT; Sigma-Aldrich; Merck KGaA) in order to identify the number of osteoclast presenting differentiation.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays. Total RNA was extracted from osteoblasts and osteoclasts in mouse femurs using an RNAeasy Mini Kit (Qiagen, Gaithersburg, MD, USA). Cyclooxygenase (COX) 2, TNF-α, NFATc1, TRAP, OSCAR, osteoclast differentiation factor (ODF) and osteoclastogenesis inhibitory factor (OCIF) mRNA expression levels in osteoblast and osteoclasts were analyzed using RT-qPCR, as previously described (27). All the forward and reverse primers were synthesized by Thermo Fisher Scientific, Inc (28). PCR amplification followed preliminary denaturation at 94˚C for 2 min, followed by 45 cycles of 95˚C for 30 sec, annealing at 57.5˚C for 30 sec, and 72˚C for 10 min in a total volume of 20 μl containing 50 ng of genomic DNA, 200 μM dNTP, 2.5 units of Taq DNA polymerase, and 200 μM of each primers. Relative mRNA expression changes were calculated using the $2^{-\Delta\Delta Cq}$ method (25). The results are expressed as the n-fold values compared with the β-actin.

Overexpression of c-Fos. Osteoclasts or osteoblasts were cultured in MEM with 5% FBS until 90% confluence and the media was subsequently removed. The c-Fos gene (GenBank: Y14808.1) was synthesized and cloned into pCMVp-NEO-X system (Takara Biotechnology Co., Ltd., Dalian, China) as described previously (29). The recombinant vector was named pCMVp-NEO-c-Fos. Cells were transfected by pCMVp-NEO-c-Fos (2 μg) using Lipofectamine 2000 (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. After 48 h, subsequent experimentations were performed.

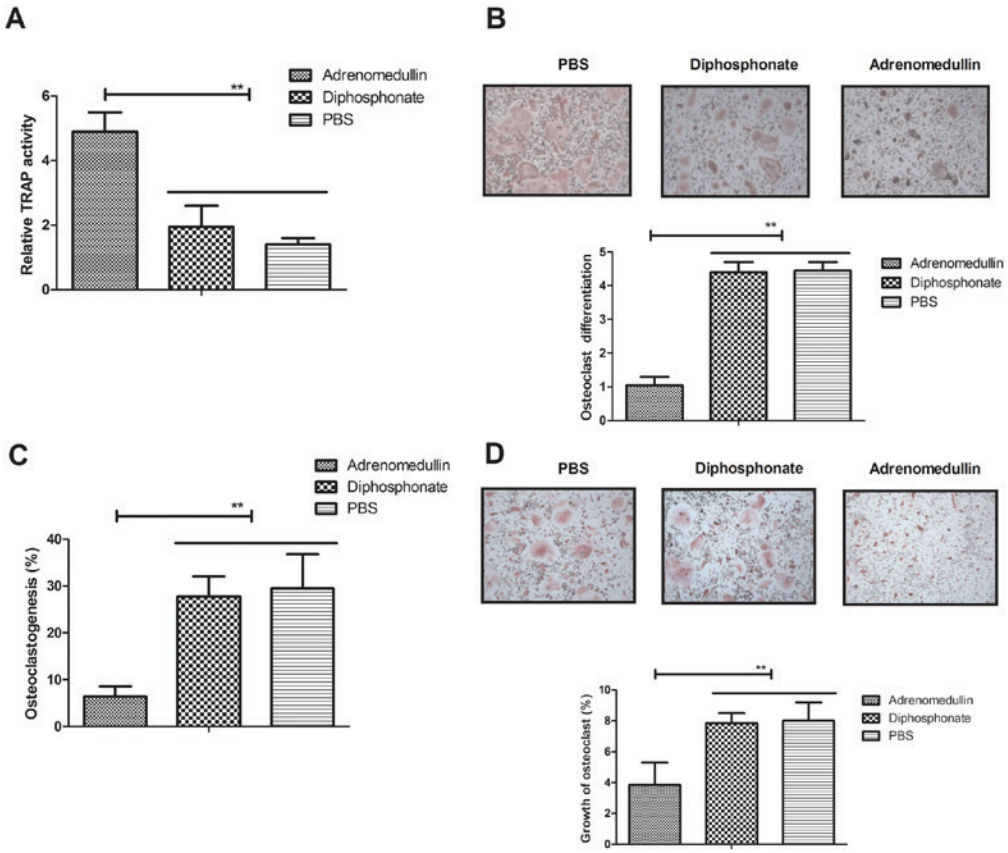

Figure 1. Adrenomedullin regulates RANKL-induced osteoclast differentiation. (A) TRAP activity following treatment with adrenomedullin, diphosphonate or PBS. (B) Analysis of osteoclast differentiation mediated by RANKL. Magnification, x400. (C) Evaluation of osteoclastogenesis following treatment with adrenomedullin, diphosphonate or PBS. (D) Growth of osteoclasts subsequent to treatment with adrenomedullin, diphosphonate or PBS, as determined by morphological analysis. Magnification, x400. Data are presented as the mean ± standard deviation. **P<0.01 vs. control. RANKL, receptor activator of nuclear factor-κB ligand; TRAP, tartrateresistant acid phosphatase; PBS, phosphate-buffered saline.

Western blot assay. Osteoblasts from experimental mice with osteoporosis treated by adrenomedullin, diphosphonate or PBS were homogenized in lysate buffer containing protease-inhibitor and centrifuged at 8,000 x g at 4˚C for 10 min. The supernatant was then used for analysis of the protein expression. For detection of the target protein, transmembrane protein were extracted using a Transmembrane Protein Extraction kit (Qiagen) according to the manufacturer's instructions. SDS-PAGE (1 μg protein) was performed as previously described (30). Protein were transferred to membranes following SDS-PAGE. Subsequent to blocking in 5% skimmed milk for 1 h at 37˚C, primary rabbit anti-mouse antibodies IκBα (1:500; cat. no. SAB4502716), p65 (1:500, cat. no. SAB4502609), IKK-β (1:1,000; cat. no. SAB4501536) and β-actin (1:2,000, cat. no. A3854; Sigma-Aldrich; Merck KGaA) were added and incubated for 1 h at 37˚C. Samples were then incubating with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used at a 1:5,000 dilution for 24 h at 4˚C. The results were visualized using a chemiluminescence detection system (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data are represented as the mean ± standard deviation, and statistical analysis was performed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). All experiments were conducted at least three times. Unpaired data were analyzed by Student's t test, while comparisons of data among multiple groups were analyzed by analysis of variance. A value of P<0.05 was considered as an indication of a statistically significant difference.

Results

Adrenomedullin suppresses RANKL-mediated differentiation of osteoclasts isolated from experimental mice. To investigate the efficacy of adrenomedullin treatment, the osteoclastogenesis was determined in the presence of RANKL and MCSF. The results indicated that TRAP activity was suppressed in osteoclasts following treatment with adrenomedullin (0.5 mg/kg) as compared with the diphosphonate-treated and PBS-treated control groups (Fig. 1A). It was also observed that adrenomedullin significantly suppressed the RANKL-mediated differentiation of osteoclasts isolated from experimental mice (P<0.01; Fig. 1B). In addition, the results shown in Fig. 1C revealed the inhibitory effects of adrenomedullin on osteoclastogenesis after 72-h RANKL stimulation, as compared with the osteoclastogenesis observed in the diphosphonate and PBS control groups. Morphological analysis also indicated that adrenomedullin suppressed the growth of osteoclasts (Fig. 1D). Taken together, these results suggest that adrenomedullin is able to inhibit RANKL-mediated osteoclast differentiation and upregulate TRAP activity in osteoclasts isolated from experimental mice.

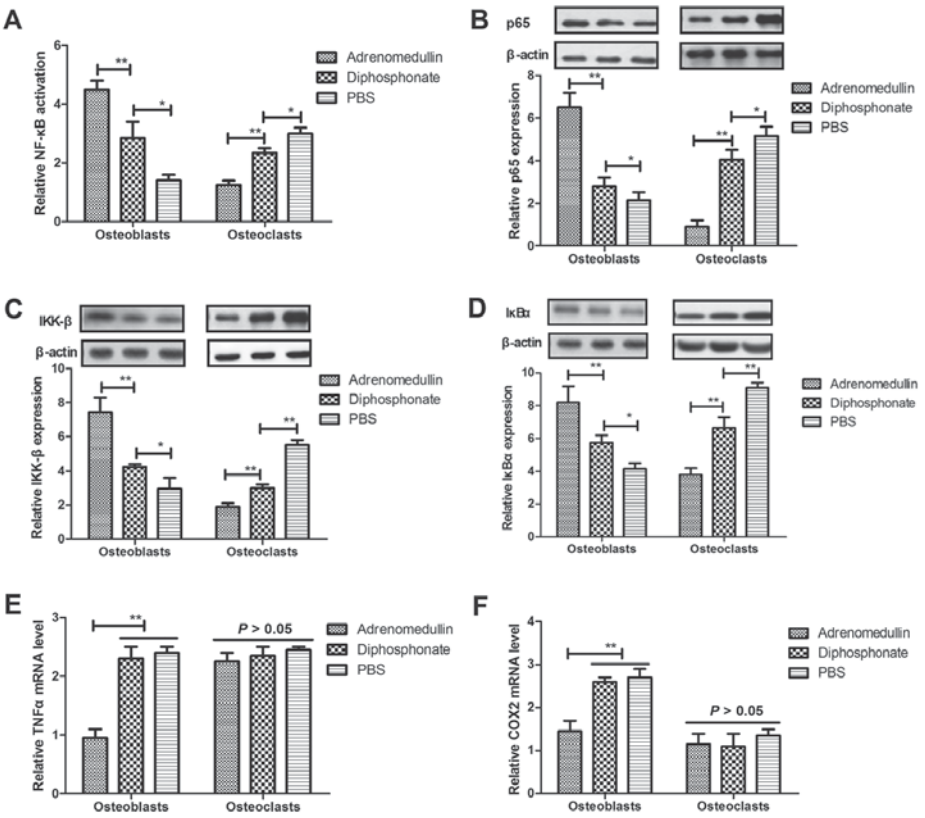

Figure 2. Adrenomedullin regulates osteoclastogenesis through regulation of NF-κB signaling pathway. (A) NF-κB activation in osteoblasts and osteoclasts following treatment with adrenomedullin. Adrenomedullin upregulated the protein expression levels of (B) p65, (C) IKK-β and (D) IκBα involved in the NF-κB signaling pathway in osteoblasts. Adrenomedullin increased the mRNA expression levels of the NF-κB target genes, (E) TNF-α and (F) COX2, in osteoblasts. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. control group. NF-κB, nuclear factor-κB; TNF-α, tumour necrosis factor-α; COX2, cyclooxygenase 2; DIP: diphosphonate; ADR, adrenomedullin (magnification, x400).

Adrenomedullin regulates RANKL-mediated osteoclastogenesis through inhibition of NF-κB activation in osteocytes. A previous study has indicated that the NF-κB signaling pathway is involved in the RANKL-mediated osteoclastogenesis (31). Therefore, the current study investigated NF-κB activation in osteocytes from experimental mice subsequent to treatment with adrenomedullin. The results demonstrated in Fig. 2A revealed that adrenomedullin upregulated the NF-κB activation in osteoblasts and downregulated the NF-κB activation in osteoclasts. Subsequently, the expression levels of several molecules in the NF-κB signaling pathway that are involved in osteoclast differentiation were investigated. As shown in Fig. 2B-D, following treatment with adrenomedullin, the p65, IKK-β and IκBα protein expression levels were significantly downregulated in osteoclasts and upregulated in osteoblasts. In addition, it was observed that the NF-κB target genes, TNF-α and COX2, were evidently decreased in the osteoblasts, while no significant effect was detected for osteoclasts (Fig. 2E and F). These results indicate that adrenomedullin treatment was able to regulate RANKL-mediated osteoclastogenesis through inhibition of NF-κB signaling pathway in osteocytes from experimental mice.

Adrenomedullin suppresses osteoclast differentiation through regulation of c-Fos expression. A previous study revealed that c-Fos is involved in osteoclast differentiation (32,33). Therefore, the present study investigated the c-Fos expression levels in osteoblasts. The expression levels of c-Fos were significantly inhibited by adrenomedullin in osteoclasts as compared with the diphosphonate and PBS control groups (Fig. 3A). However, overexpression of c-Fos activity suppressed the adrenomedullin-mediated NF-κB activity in osteoclasts (Fig. 3B). Next, the NFATc1, TRAP and OSCAR expression levels in osteoclasts following treatment with adrenomedullin were analyzed. The results shown in Fig. 3C-E revealed that NFATc1, TRAP and OSCAR expression levels were decreased in osteoclasts following treatment with adrenomedullin. However, no regulatory effects of adrenomedullin on osteoblasts were observed in the present study. Furthermore, the results illustrated in Fig. 3F-G revealed that adrenomedullin markedly reduced the gene expression levels of ODF and OCIF in osteoclasts. The aforementioned indicated that adrenomedullin inhibited osteoclast differentiation through regulation of the c-Fos-mediated NF-κB signaling pathway in osteoclasts or osteoblasts.

Adrenomedullin prevents the progression of glucocorticoid-induced osteoporosis in vivo. Subsequent to analysis of the *in vitro* effects of adrenomedullin on osteoclast differentiation, the *in vivo* efficacy of adrenomedullin for the treatment of glucocorticoid-induced osteoporosis was further examined. As expected, adrenomedullin treatment markedly decreased the bone mass loss of mice with glucocorticoid-induced osteoporosis as determined by

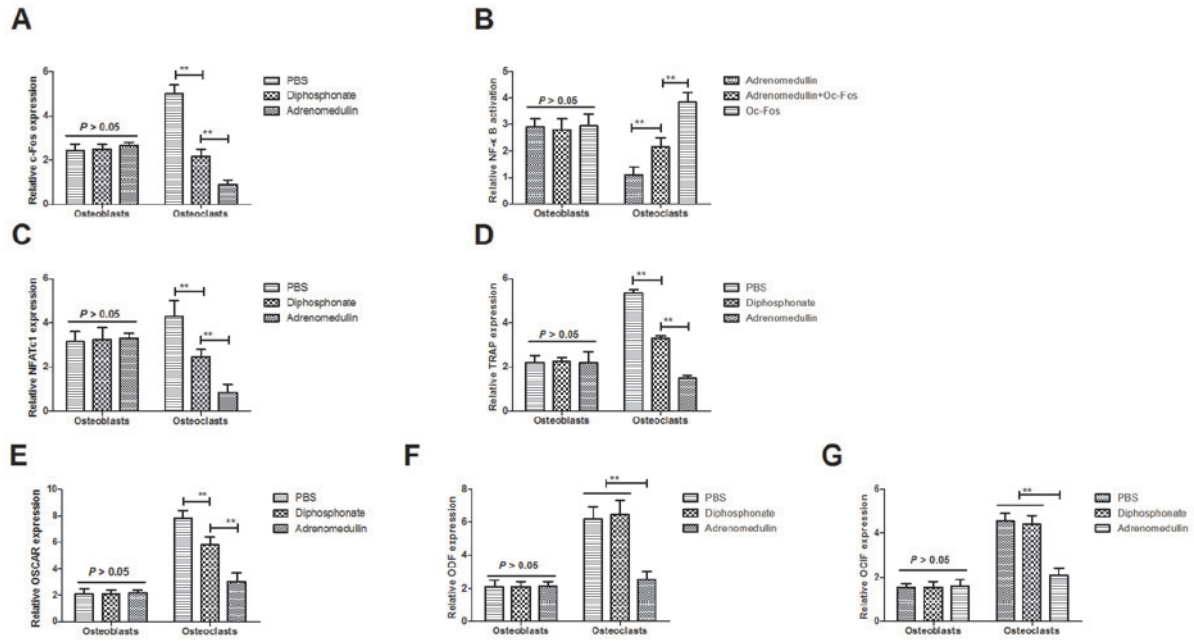

Figure 3. Expression of c-Fos is involved in adrenomedullin-mediated osteoclast differentiation. (A) Expression levels of c-Fos in osteoclasts following treatment with adrenomedullin, diphosphonate or PBS. (B) Inhibition of c-Fos expression suppresses the adrenomedullin-mediated NF-κB activity. (C) NFATc1, (D) TRAP and (E) OSCAR mRNA expression levels in osteoclasts, following treatment with adrenomedullin, diphosphonate or PBS. Gene expression levels of (F) ODF, and (G) OCIF in osteoclast after treatment with adrenomedullin, diphosphonate or PBS. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. control group. NF-κB, nuclear factor-κB; NFATc1, NF of activated T-cells 1; TRAP, tartrate-resistant acid phosphatase; OSCAR, osteoclast-associated immunoglobulin-like receptor; ODF, osteoclast differentiation factor; OCIF, osteoclastogenesis inhibitory factor.

immunostaining assay (Fig. 4A). The femur bone density of mice with glucocorticoid-induced osteoporosis was improved following treatment with adrenomedullin when compared with the controls (Fig. 4B). Furthermore, the maximum load, stiffness, energy to failure, ultimate strength, elastic modulus, and post-yield displacement were also increased in mice after treatment with adrenomedullin (Fig. 4C-H). These findings demonstrated that adrenomedullin exerted a beneficial effect against glucocorticoid-induced osteoporosis in mice by regulation of the bone mineral density and bone strength *in vivo*.

Discussion

Previously, RANKL-mediated osteoclastogenesis and osteoclast differentiation have been demonstrated to serve a crucial role in the progression and development of osteoporosis (34,35). In addition, studies have indicated that adrenomedullin is a multifunctional protein that is associated with disease progression in patients with primary osteoporosis (36). Furthermore, the efficacy of adrenomedullin on osteoclast differentiation has been examined in previous studies, which suggested that adrenomedullin efficiently inhibited the differentiation of osteoclasts (37,38). In the current study, in order to better understand the association between adrenomedullin treatment and osteoporosis, the molecular mechanism of adrenomedullin-mediated osteoclastogenesis and osteoclast differentiation was investigated in mouse osteoclasts and in glucocorticoid-induced osteoporosis mice. The present study also analyzed the c-Fos-induced osteoclast differentiation after treatment with adrenomedullin. The results revealed that adrenomedullin regulated RANKL-mediated osteoclastogenesis through inhibition of NF-κB activation in osteocytes *in vitro* and *in vivo*. Notably, the current findings presented that adrenomedullin treatment not only inhibited the osteoclastogenesis and osteoclast differentiation, but also improved the bone quality of mice with glucocorticoid-induced osteoporosis *in vivo*. These results suggest that adrenomedullin may be an efficient drug for the treatment of glucocorticoid-induced osteoporosis.

The functions of osteoclasts include bone resorption and bone dynamic equilibrium, while they are also involved in boneassociated disorders (39,40). Numerous studies have reported the regulatory effects of osteoclasts in the progression of osteoporosis. It has been indicated that inhibition of osteoclast differentiation improves osteoporosis (41,42). In addition, osteoclast activity is upregulated during osteoporosis, and regulation of osteoclast activity may be an effective target for clinical treatment of osteoporosis (43,44). Although several studies have investigated the efficiency of various drugs for the differentiation and function of osteoclasts by regulating differentiation, the actual molecular signaling pathway has seldom been reported (45-48). The present study investigated the cellular mechanism underlying the adrenomedullin-mediated osteoclast differentiation and bone resorption in a mouse model of osteoporosis. Despite the consistency of the current results with previous findings reported in the literature (38), the present study also suggested that adrenomedullin regulated the activities of osteoclasts through NF-κB activation in osteocytes *in vitro* and *in vivo*.

To date, numerous studies have been conducted attempting to understand the adrenomedullin-associated

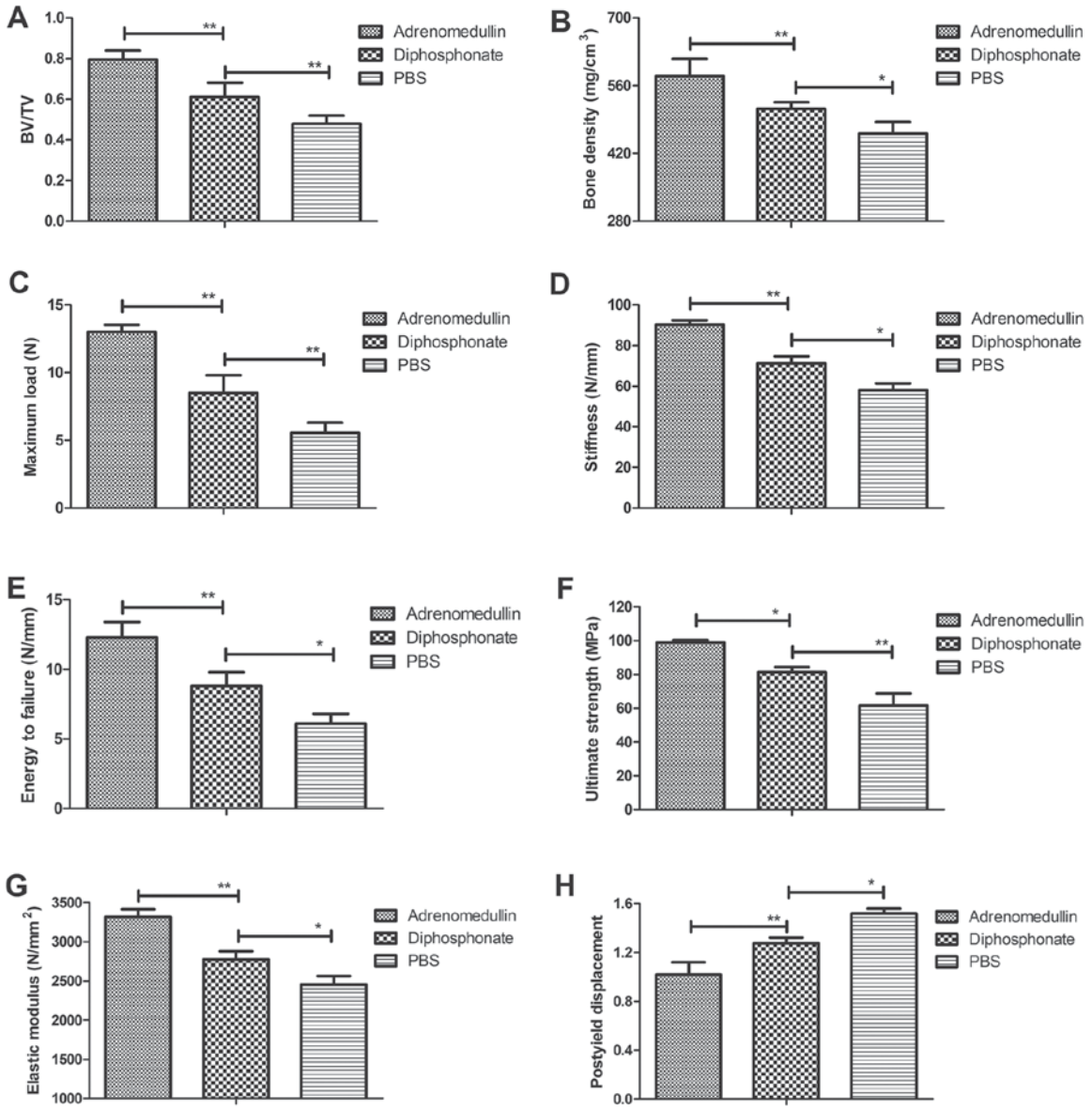

Figure 4. *In vivo* efficacy of adrenomedullin in the disease progression of mice with glucocorticoid-induced osteoporosis. (A) Bone mass loss in mice with glucocorticoid-induced osteoporosis was determined by immunostaining assay. (B) Femur bone density in mice with glucocorticoid-induced osteoporosis following treatment with adrenomedullin, diphosphonate or PBS. Detection of changes in the (C) maximum load, (D) stiffness, (E) energy to failure, (F) ultimate strength, (G) elastic modulus and (H) post-yield displacement following treatment. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. control group. BV/TV, bone volume/total volume.

therapeutic regimen via targeting specific molecules and disrupting the dopaminergic system, leading to various syndrome symptoms of osteoporosis (49,50). Although previous study demonstrated the prevention of bone loss in a model of postmenopausal osteoporosis through adrenomedullin inhibition (37), the mechanism underlying the adrenomedullin-inhibited osteoclast differentiation has not been elaborated. In addition, Pleguezuelos *et al* (51) have demonstrated that the NF-κB signaling pathway was involved in the adrenomedullin-induced function in multiple biological processes. Furthermore, the role of NF-κB on the production and secretion of adrenomedullin has been investigated in a previous study (52). Lee *et al* (53) have suggested that NF-κB activation is associated with the osteoclast differentiation and that downregulation of RANKLinduced osteoclast differentiation through inhibition of IkB degradation. In the current study, it was assumed that adrenomedullin regulated osteoclast activity via the NF-κB signaling pathway. The present results confirmed this hypothesis and indicated that adrenomedullin exerted a beneficial effect in mice with glucocorticoid-induced osteoporosis. These findings identified that the maximum load, stiffness, energy to failure, ultimate strength, elastic modulus and post-yield displacement were improved following treatment with adrenomedullin.

In conclusion, the results of the present study revealed that adrenomedullin exerts inhibitory effects on RANKLinduced osteoclastogenesis and differentiation of osteoclasts via inhibition of the NF-κB signaling pathway, as well as inhibits cFos degradation in osteoblasts. Therefore, it is suggested that adrenomedullin may be identified as a potential candidate agent for the treatment of RANKLinduced osteoclast differentiation and glucocorticoid-induced osteoporosis.

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