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ORIGINAL ARTICLE

Down-regulation of N-methyl D-aspartate receptor in rat-modeled disuse osteopenia

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Abstract Lack of mechanical stress may result in osteoporosis; however, the underlying mechanisms of disuse osteoporosis remain unclear. It has been indicated that mechanical loading causes extracellular glutamate accumulation in osteoblasts. We hypothesized that the glutamate receptor mediation on bone cells might also be involved in mechanically stimulated osteogenesis. In this study, we investigated the changes of bone formation and the expressions of osteogenic genes and N-methyl D-aspartate (NMDA) receptors, the major glutamate receptors, in disused bones. Rat modeled disuse osteopenia in hind limbs was induced by a 3-week tail suspension in Sprague-Dawley rats. Bone mineral density and trabecular bone volume of distal femurs were measured to verify the osteopenia of disused bones. The mRNA expressions of cbfa1/Runx2, type I collagen, alkaline phosphatase (ALP) and osteocalcin (OC) in bones were measured as osteogenic markers. The influences of mechanical unloading on the expressions of NMDA receptors (NR_1 and NR_2D) in bones were also examined. The effects of NMDA mediation on osteogenesis were tested by a treatment of MK-801, a non-competitive NMDA receptor antagonist, in cultured osteoblasts and bone marrow stroma cells. Our result showed that mRNA expressions of cbfa1/Runx2, type I collagen, ALP and OC were significantly decreased in disused bones. The mRNA and protein expressions of NR_1 and NR_2D were significantly decreased in disused bones; furthermore, immunolocalization of both receptors showed decreases in osteoblasts, but not in osteoclasts. The results from the in vitro study showed that MK-801 inhibited mRNA expression of cbfa1/Runx2 in bone marrow stroma cells and also inhibited those of collagen type I, ALP and OC of osteoblasts in a dose-dependent manner. These results suggest that NMDA receptor mediation may play an important role in transmitting mechanical loading in bones, and decreases of the expressions of NMDA receptors in disused bones, especially in osteoblasts, may contribute to the decrease of osteogenesis.

Keywords Bone formation · Disuse osteoporosis · NMDA receptor · Osteoblasts

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Introduction

Mechanical stimuli contribute to the balance of bone formation and resorption during the normal remodeling process [1]. It has been reported that a reduction of mechanical stress on bone inhibits osteoblast-mediated bone formation, accelerates osteoclast-mediated bone resorption and leads to disuse osteoporosis [2]. Skeletal unloading in a tail-suspension rodent model demonstrated that bone formation was suppressed, and the proliferation and osteogenic potential of bone marrow stroma cells and differentiated osteoblasts were also decreased [3, 4, 5, 6, 7]. On the other hand, osteoclast number and activity in trabecular bones were reported to be increased in a disuse state [5]. However, the underlying molecular mechanisms mediating the changes of bone cell functions by unloading remain unclear.

A previous report indicated that mechanical loading down-regulated the expression of a glutamate/aspartate

transporter (GLAST) in osteoblasts and osteocytes [8]. Osteoblasts and osteoclasts were also reported to possess N-methyl D-aspartate (NMDA) receptors, the major glutamate receptors, and mechanical loading increased the expressions of these receptors [9, 10]. Blockade of glutamate receptors caused down-regulation of osteogenic markers, type I collagen, alkaline phosphatase, osteocalcin and mineralization in cultured osteoblasts [11, 12]. On the other hand, blockade of NMDA receptors by MK801 was also reported to inhibit bone resorption [13]. This NMDA receptor mediation was found to affect the differentiation of osteoclast precursors, but not the viability of mature osteoclast [13, 14]. Accordingly, glutamate release, uptake and/or receptor function may play an important role in the regulation of bone remodeling. However, it is not clear whether the glutamate receptor mediation is involved in the mechanisms of bone loss in disuse osteoporosis.

In this study, we hypothesized that mechanical unloading might alter the expressions of glutamate receptors and subsequently affect the functional gene expressions of osteoblastic and/or osteoclastic linage, and thus contribute to bone loss in a disuse state. We examined the changes of bone formation histologically, and the gene expressions of osteogenic markers and NMDA receptors in disused bones. The influence of mechanical unloading on the expressions of NMDA receptors in osteoblasts and osteoclasts residing near the trabecular bones was also observed histologically in disused bones. Blockade of NMDA receptors was also performed to test the influences of glutamate signaling on the expressions of osteogenic genes in cultured bone marrow stroma cells and calvaria-derived osteoblasts.

Materials and methods

Experiment animals

Rat-modeled disuse osteoporosis was induced by a 3-week tail suspension in 3-month-old male Sprague-Dawley rats. By suspending the tail, the hind limbs of a rat were elevated to keep them from touching the floor, and were in a disuse state. Rats housed in the same condition without tail suspension were used as the control animals. After 3 weeks, the control and tail-suspended rats were used for experiments. After experiments, all the rats were killed by an overdose of CO_2. Femurs were removed for histological study and extracted for specific mRNA and protein detections.

Bone mineral density (BMD)

BMD of femurs were measured by a dual energy X-ray absorption meter (Norland XR-36; Norland, Fort Atkinosin, Wis.). BMD (g/cm^2) was the quotient of bone mineral content divided by projectional bone area.

Histology

Histomorphometry

Rat femurs were decalcified in a 24% disodium EDTA/PBS (phosphate buffered solution) at pH 7.4 for 3 weeks following a fixation in 4% paraformaldehyde solution. After paraffin embedding, the femurs were dissected at a 5-μm thickness. Bone sections were hematoxylin and eosin (H&E) stained for histomorphometry of trabecular bone volume. Ratios of trabecular bone volume and total volume (BV/TV) of distal femurs were measured by counting the trabecular volume in five random areas (4.613419 mm^2/area) of the central metaphysis starting 1 mm from the epiphyseal growth plate [15] by using Image-Pro Plus analysis software (Media Cybernetics, Sliver Spring, Md.).

Immunolocalization of NMDA receptor

Functional subunit proteins of the NMDA receptor, NR_1 and NR_2D, were immunolocalized in bone sections. Bone sections were boiled in 10 mmol/l sodium citrate (pH 6.0) for 5 min to expose the antigen epitope. The endogenous peroxidase of sections was eliminated by incubating with a 3% hydrogen peroxide/methanol solution for 10 min. After incubating with a blocking solution for 1 h at room temperature, sections were then bound with NR_1 or NR_2D antibody (1:300 or 1:500, respectively) for another 2 h at 37°C in a humid chamber. After washing, sections were incubated with anti-mouse biotinated secondary antibody for 20 min and then streptoavidin-peroxidase for another 20 min. Color was developed by a 3,3'-diaminobenzidine solution containing 0.01% hydrogen peroxide [16]. Bone sections were counterstained by hematoxylin. Ratios of immunostained and hematoxylin-stained cells were measured in central metaphysis in a total of 500 counted osteoblasts or osteoclasts from five random areas. The mononuclear cells residing around the surface of trabecular bones were counted as osteoblasts, while the polynuclear cells were counted as osteoclasts.

Isolation and culture of bone marrow stroma cells (BMSC)

Bone marrow stroma cells (BMSC) were isolated from 6–7-week old Sprague-Dawley rats. After the rats were killed by an overdose of CO_2, the tibias were cut transversally at the epiphyseal area. Bone marrow was flushed out with PBS and collected into Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS). Cell suspension was applied to

a 70% Percoll gradient, and the bone marrow stroma cell-enriched low-density fraction was collected for culture. Cells were cultured in DMEM containing 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin in a 25-T flask, and the medium was changed every 2 days. The second subcultured cells were detached and seeded at 5,000 cells/cm^2 in a 10-cm dish for experiments. The influence of NMDA receptor mediation on osteoblastogenesis of BMSC was tested by treatment with a noncompetitive NMDA receptor antagonist, MK-801, a channel antagonist dizocilpine. MK-801 has been used in cultured osteoblasts and osteoclasts at 10^{-6}-10^{-4} M, and revealed no significant cytotoxicity on cells [14, 17, 18, 19]. Cells reaching sub-confluence were treated with MK-801 (10^{-6}-10^{-4} M) for 6 days. After treatment, cells were harvested and mRNA was extracted for measuring the expression of cbfa1/Runx2.

Isolation and culture of calvarial osteoblasts

Primary calvarial osteoblast cultures were prepared from parietal bones obtained from fetal Sprague-Dawley rats of 21-day gestation. The mother rat was killed under anesthesia; intact uteri were removed and transferred into a sterilized dish. Fetal rats were removed from the uteri, and the fetal calvaria were isolated. Parietal bones were dissected free from the sutures. The periosteal layers from both sides of bones were carefully removed. The bones were cut into chips and washed with sterilized Hanks' buffered solution. Cells were released from the bone chips by five 20-min sequential collagenase digestions (Worthington Corp, Freehold, N.J.) [20, 21]. The last three digestions, in which cells expressed mature osteoblasts phenotypes, were pooled to provide an osteoblast cell suspension [22]. Cells were cultured in DMEM containing 100 μg/ml ascorbic acid, non-essential amino acid, penicillin/streptomycin and 10% fetal calf serum (Gibco-BRL, Grand Island, N.Y.). Cultures were incubated in a 37°C, 5% CO_2 incubator. Medium was changed every 2 days. Osteoblasts were cultured in a medium with or without MK-801 (10^{-6}-10^{-4} M) for 7, 14 or 21 days. The 7-day cultures represented the osteoblasts in the proliferation phase, while the 14- and 21-day cultures represented osteoblasts in matrix maturation and mineralization phases, respectively. Osteoblastic mRNA expression of type I collagen, alkaline phosphatase (ALP) or osteocalcin from the 7-, 14- or 21-day cultures was measured, respectively.

Transcription-polymerase chain reaction (RT-PCR)

Total RNA from bones, cultured BMSC or osteoblasts was extracted by using the TRI reagent (Life Technologies, Grand Island, N.Y.). The mRNA of NR_1, NR_2D, cbfa1/Runx2, collagen type I, ALP, osteocalcin was measured by RT-PCR. The first strand cDNA was converted from 5 μg of RNA by Moloney murine leukemia virus RT and oligo(dt) primer. PCR was performed with an Applied Biosystems GeneAmp 9,600 PCR system (Applied Biosystems, Foster, Calif.). The PCR reaction was carried out with the specific primers of each gene. The primer sequences were as follows: NR_1 (333 bp product): 5' gct tga tga gca ggt cta tgc 3'; 5' aat gac ccc agg ctc aga aac 3'; NR_2D (179 bp product): 5' tta ctc gag ccc cgc cac aca gaa a 3; 5' aca ctc gag ggt agg act ttc gtg 3'; Procollagen I1α (502 bp product): 5' taa agg gtc atc gtg gct tc 3'; 5' act ctc cgc tct tcc agt ca 3'; ALP (499 bp product): 5'ctc cgg atc ctg aca aag aa 3'; 5'acg tgg ggg atg tag ttc tg 3'; Cbfa1/Runx2 (216 bp product): 5' gaa gag gct gtt tga cgc cat 3'; 5' cgg tgc aaa ctt tct cca gg 3'; Osteocalcin (698 bp product): 5' cct gac tgc att ctg cct ctc 3'; 5' tcc gct agc tcg tca caa ttg g 3';.

The product of PCR was resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide. The β-actin or GAPDH (glyceraldehydes-3-phosphate dehydrogenase) mRNA was also amplified as a housekeeping to normalize the loading difference.

Western blot analysis

The protein levels of NR_1 and NR_2D in rat distal femur were measured by Western blot analysis. Cell membrane protein was prepared in the same manner as in the previous description [23]. Briefly, bone samples were homogenized in dissection buffer (50 mM Tris acetate, pH 7.4, 10% sucrose, 5 mM EDTA) and centrifuged at 16,000 × g for 30 min. The pellet was stored at −70°C for protein analysis. The protein concentration was measured [24], and an equal amount of each sample protein was separated on a 7.5% SDS-polyacrylamide gel. The gel was transferred onto polyvinylidene difluoride (PVDF) transfer membrane (NEN Life Science, Boston, Mass.) by electroblotting for 1 h. The membrane was blocked overnight at 4°C with the Tween-Tris buffer saline solution (t-TBS; 20 mM Tris base, 0.44 mM NaCl, 0.1% Tween 20, pH 7.6) containing 5% non-fat dry milk. After blocking, the membrane was incubated with NR_1 monoclonal antibody (Pharmingin, San Diego, Calif.) at a 1:500 dilution or NR_2D polyclonal antibody (Santa Cruz, Santa Cruz, Calif.) at a 1:500 dilution in t-TBS containing 5% non-fat milk for 2 h, and then incubated with goat HRP conjugated anti-mouse IgG (1:2,000) (Santa Cruz, Santa Cruz, Calif.) for another 1 h. β-tubulin was also measured as a housekeeping [25]. The immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham, Piscataway, N.J.) [26].

Statistics

Data compared between the control and hind limb disused groups were analyzed by Student's *t*-test.

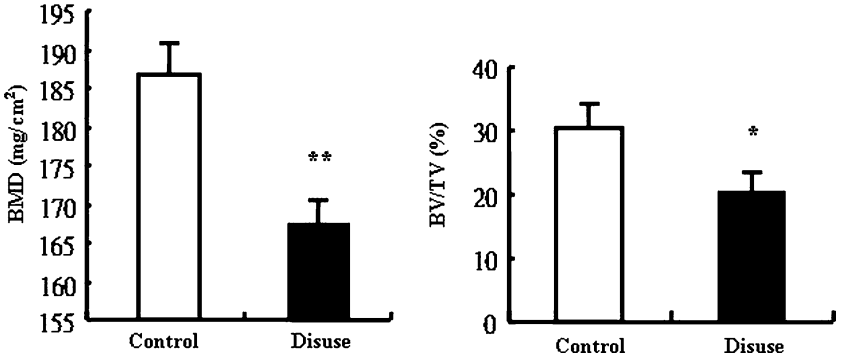

Fig. 1 Decreases of bone mineral density (*BMD*) and trabecular bone volume in a 3-week tail suspension modeled rat disuse osteopenia. *Left panel*: BMD (mg/cm^2) was measured by dual energy X-ray. *Right panel*: ratios of trabecular bone volume and total volume (*BV/TV*) of distal femurs were measured by counting the trabecular volume in five random areas (4.613419 mm^2/area) of the central metaphysis starting 1 mm from the epiphyseal growth plate. *Bars* represent mean ± SE of six samples. Experiments were repeated at least three times with similar results. Data were evaluated by Student's *t* -test. * $P < 0.05$; ** $P < 0.01$ in comparison with the control group

Comparisons of data from dose response effects of MK-801 on mRNA expressions were analyzed by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. $P < 0.05$ was considered to be statistically significant.

Results

BMD and histomorphometry

BMD and BV/TV of distal femurs from tail-suspended rats were significantly lower than those of the control rats (Fig. 1). This result indicated a 3-week hind limb unloading certainly induced osteopenia in the study.

The mRNA expressions of osteogenic markers in bones

In comparison to the control rats, mRNA expressions of cbfa1/Runx2, collagen type I, ALP and osteocalcin in the extracts of distal femurs were significantly decreased in the tail-suspended rats (Fig. 2A–D).

The mRNA expressions and subunit protein levels of NMDA receptor in bones

In comparison to the control rats, both the mRNA expressions and the protein levels of NR_1 and NR_2D, subunit proteins of the NMDA receptor, in the extract

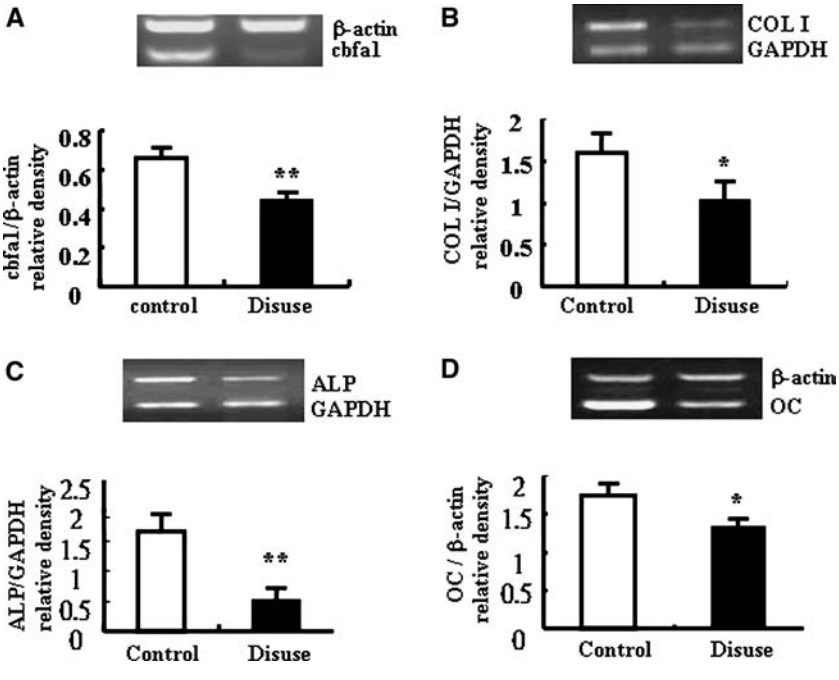

Fig. 2 Decreases of mRNA expressions of osteogenic genes in a 3-week tail suspension modeled rat disuse osteopenia. The mRNA expressions of osteogenic genes from distal femurs were detected by PT-PCR. Representative PT-PCR images of (**A**) cbfa1/Runx2 (*cbfa1*), (**B**) collagen type I (*COL I*), (**C**) alkaline phosphatase (*ALP*) and (**D**) osteocalcin (*OC*), and housekeeping genes, β-actin and GAPDH, are shown. The ratios of the relative optical density of each target gene to that of a housekeeping gene were compared among the control and disuse groups. Each *bar* represents mean ± SE of six samples. Experiments repeated at least three times with similar results. Data were evaluated by Student's *t* -test. *$P < 0.05$; ** $P < 0.01$ in comparison with the control group

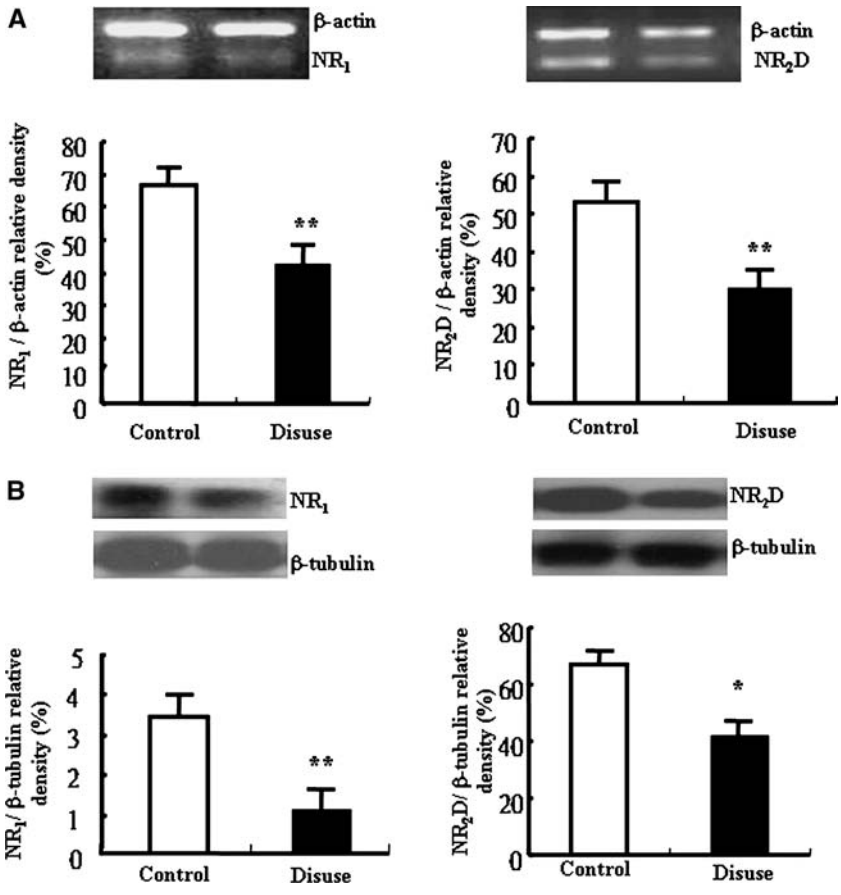

Fig. 3 Down-regulations of mRNA and protein of the subunits of NMDA receptor in a 3-week tail suspension modeled rat disuse osteopenia. Representative images of NR_1, NR_2D and housekeeping genes, β-actin and β-tubulin, from PT-PCR (**A**) and Western blot analysis (**B**) are shown. The ratios of the relative optical density from the band of each target gene to that of a housekeeping gene were compared among the control and disuse groups. Each *bar* represents mean ± SE of six samples. Experiments repeated at least three times with similar results. Data were evaluated by Student's *t* -test. $*P < 0.05$; $**P < 0.01$ in comparison with the control group

of distal femur were significantly lower in the tail-suspended rats (Fig. 3). The result of immunohistochemistry on bone sections from distal femurs showed that the ratio of either NR_1 or NR_2D immuno-stained osteoblasts to the total counted osteoblasts was significantly lower than that of the control rats (Fig. 4). However, the ratio of neither NR_1 nor NR_2D immuno-stained osteoclasts to the total osteoclasts showed any significant difference between the control and tail-suspended groups (Fig. 4).

The mRNA expressions of osteogenic markers decreased by NMDA blockade in bone marrow stroma cells and calvaria osteoblasts

Upon treatment with MK801 (10^{-6}-10^{-4} M) for 6 days, mRNA expression of cbfa1/Runx2 in cultured bone marrow stroma cells was significantly decreased (10^{-6} M and 10^{-5} M, $P < 0.05$; 10^{-4} M, $P < 0.01$) in a dose-dependent manner (Fig. 5). The mRNA expression of collagen type I from the 7-day cultured osteoblasts was significantly decreased by MK801 (10^{-6}-10^{-4} M) ($P < 0.01$). ALP mRNA expression from the 14-day cultured osteoblasts was also decreased by MK801 at 10^{-5}–10^{-4} M ($P < 0.05$). Osteocalcin mRNA from 21-day cultured osteoblasts was also suppressed by MK801 (10^{-6} and 10^{-5} M, $P < 0.05$; 10^{-4} M, $P < 0.01$) (Fig. 6).

Discussion

In this study, we used a 3-week tail suspension to induce disuse osteopenia in rats and demonstrated the decreases of BMD, trabecular volume and osteogenic gene expressions in disused bones. More importantly, we found that the mRNA and protein expressions of functional subunits of the NMDA receptor, NR_1 and NR_2D were also decreased in the extract of disused bones. Furthermore, we found a decrease of the ratio of osteoblasts possessing NR_1 and NR_2D to the total osteoblasts residing in the trabeculae of disused bones, but that of the osteoclasts remained unchanged. These results indicated that mechanical unloading down-regulated the expression of the NMDA receptor in osteoblasts rather than osteoclasts. Previous reports indicated that a disused state increases the osteoclast-related bone resorption [13], and other studies found that NMDA receptor signaling is involved in the differentiation of preosteoclasts, but not the viability of mature osteoclasts [13, 14]. Our in vivo study further demonstrated that the expressions of NMDA receptors in mature osteoclasts were not affected by mechanical unloading.

Previous reports have demonstrated that several growth factors, such as IGF-I and TGFβ2, and also osteogenic markers such as osteopontin and osteocalcin,

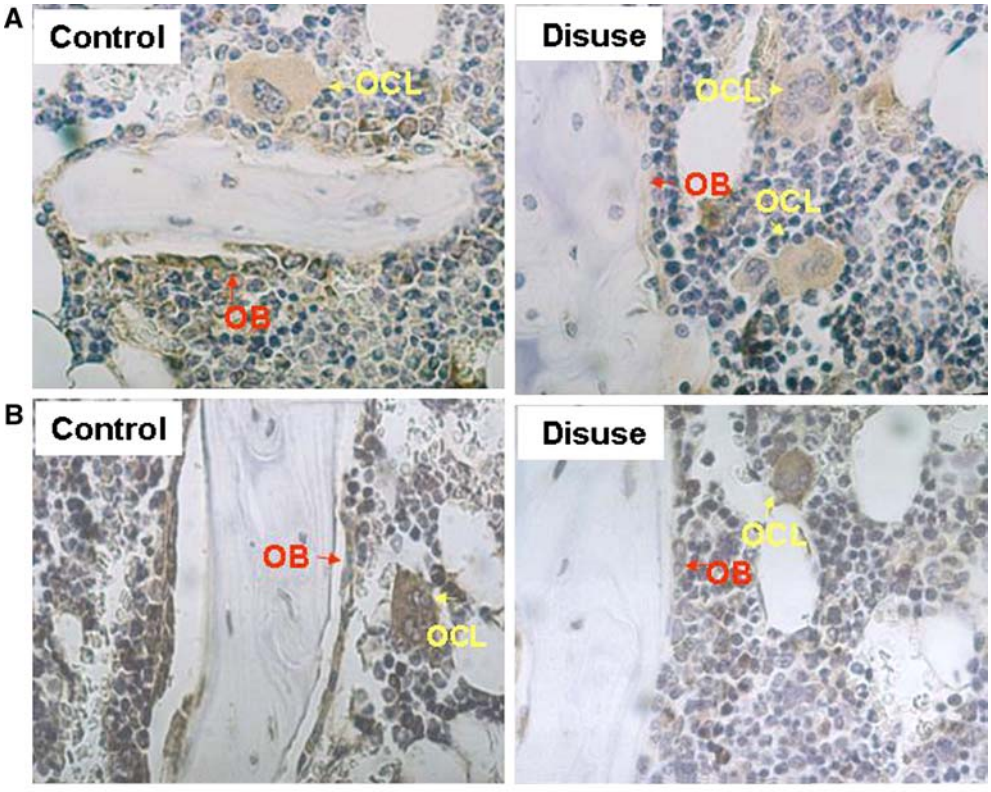

Fig. 4 Immunolocalization of subunit proteins of the NMDA receptor on bone sections from distal femurs. Representative micrographs from immunohistochemistry of NR_1 (**A**) and NR_2D (**B**) are shown (magnification ×400). Bone sections from distal femurs were immuno-stained with NR_1 or NR_2D antibody (*brown color*) and counterstained with hematoxylin (*blue color*). Ratios of immuno-stained and hematoxylin-stained osteoblasts or osteoclasts (NR_1/total or NR_2D/total) were measured at central metaphysis in a total counted 500 osteoblasts or osteoclasts from five random areas. Data are shown as mean ± SE of six samples (*lower panel*). Data from osteoblasts or osteoclasts of the control and disuse groups were compared separately, and evaluated by Student's *t* -test. Experiments repeated at least three times with similar results. * $P < 0.05$ in comparison with the control group

	Osteoblast		Osteoclast	
	Control	Disuse	Control	Disuse
%NR_1/total (mean± SE)	87.9± 2.5	70.3±4.7*	85±3.8	81.8±2.8
%NR_2D/total (mean± SE)	86.4±1.0	83.9±0.1*	97.6±1.4	91.1±0.4

are coincidently down-regulated in unloaded bones of immobilized animals [27, 28, 29]. It was suggested that mechanical unloading might decrease osteogenic markers through altering the productions of these growth factors in unloaded bones [29]. This growth factor-associated suppression of osteoblastic functions in unloaded bones was reported to only happen in the early stage (4–7 days), but not in the late stage (14 days) of immobilization [29]. A previous report indicated that during mechanical unloading, the increases of osteoclastic functions occurred prior to the decreases of osteoblastic functions, and thereafter both functional changes were sustained for a long duration (21 days) in immobilized bones [30]. Accordingly, the regulatory mechanisms of mechanical stimulus-mediated bone remodeling would be regulated by complicated temporal and spatial factors. It was also reported that the intracellular Ca^{+2} influx plays an important role in the conversion of mechanical stimuli into biological response in bone cells [31, 32, 33]. A recent report indicated that the long-lasting voltage-sensitive calcium channels mediate the mechanically induced bone formation [34]. On the other hand, neural glutamate transporters were found to be down-regulated in loaded bones, but not in unloaded bones [8]. Decreases of glutamate transporters, resulting in glutamate accumulation in mechanically stimulated bones, suggested that glutamate might play a role in paracrine intercellular communication in bones [8]. This evidence raises the possibility that glutamate mediation may be involved in the conversion of mechanical stimulus to osteogenic response in bone cells. Our results from this study showed that the NMDA receptors decrease in osteoblasts, but not osteoclasts, of disused bones after 3 weeks of mechanical unloading, indicating that a decrease of NMDA signaling in osteoblasts may be involved in the decline of bone formation in a long-term disuse state.

The results from this in vitro study showed that NMDA receptor blockade decreased the expression of cbfa1/Runx2 in bone marrow stroma cells and also de-

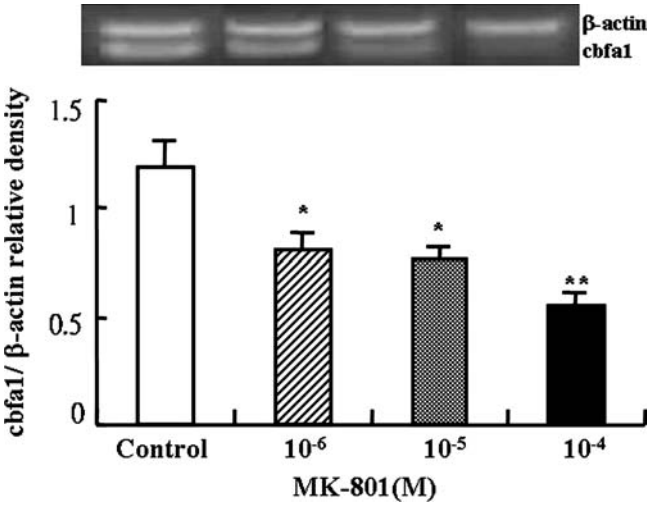

Fig. 5 NMDA receptor blockade down-regulated cbfa1/Runx2 expression in cultured bone marrow stroma cells. Representative PT-PCR images of cbfa1/Runx2 (cbfa1) and β-actin from bone marrow stroma cells are shown. The ratios of the relative optical densities from the band of cbfa1/Runx2 to that of β-actin were compared among cultures treated with different concentrations of MK-801. Each *bar* represents mean ± SE of six replicated cultures. Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. * $P < 0.05$; ** $P < 0.01$ in comparison with the control group

creased the expressions of collagen type I, ALP and osteocalcin in various differentiation stages of mature calvarial osteoblasts. Although previous studies have indicated NMDA signaling increased the osteogenic functions [11, 12], our results further elucidated that NMDA mediated the differentiations during bone progenitors to osteoblasts (osteoblastogenesis) and the maturation of osteoblasts. Together with the results of our in vitro and in vivo studies, we suggest that mechanical unloading caused osteopenia may occur partly by inhibiting the expressions of NMDA receptors in bone marrow stroma cells and osteoblasts, and thus decreases the expressions of the key osteogenic genes in these cells.

Cbfa1/Runx2 is a transcription factor that specifically binds to the OSE2 (osteoblast-specific cis-acting element 2) in the promoter of OG2 (osteocalcin gene 2) and regulates the expression of osteocalcin. It has been suggested that in addition to osteocalcin, cbfa1 might also regulate the expression of the key differentiation markers of osteogenesis such as collagen type I, osteopontin, bone sialoprotein, alkaline phosphatase, etc. [35]. Previous studies have indicated that cbfa1/Runx2 was phosphorylated and activated by mitogen-activated protein kinase (MAPK) [36], and mechanical loading activated the DNA binding activity of cbfa1/Runx2 through the MAPK pathway [37]. It implies that mechanical loading stimulates bone formation, at least partly, by activating the transcriptional function of cbfa1/Runx2. On the other hand, mechanic loading was also reported to stimulate osteoblastic function by the downstream effectors, activator protein-1 (AP-1), through MAPK and Rho kinase [38]. It is quite possible that AP-1 could regulate cbfa1 expression since the cbfa1/Runx2 promoter region possesses AP-1 binding

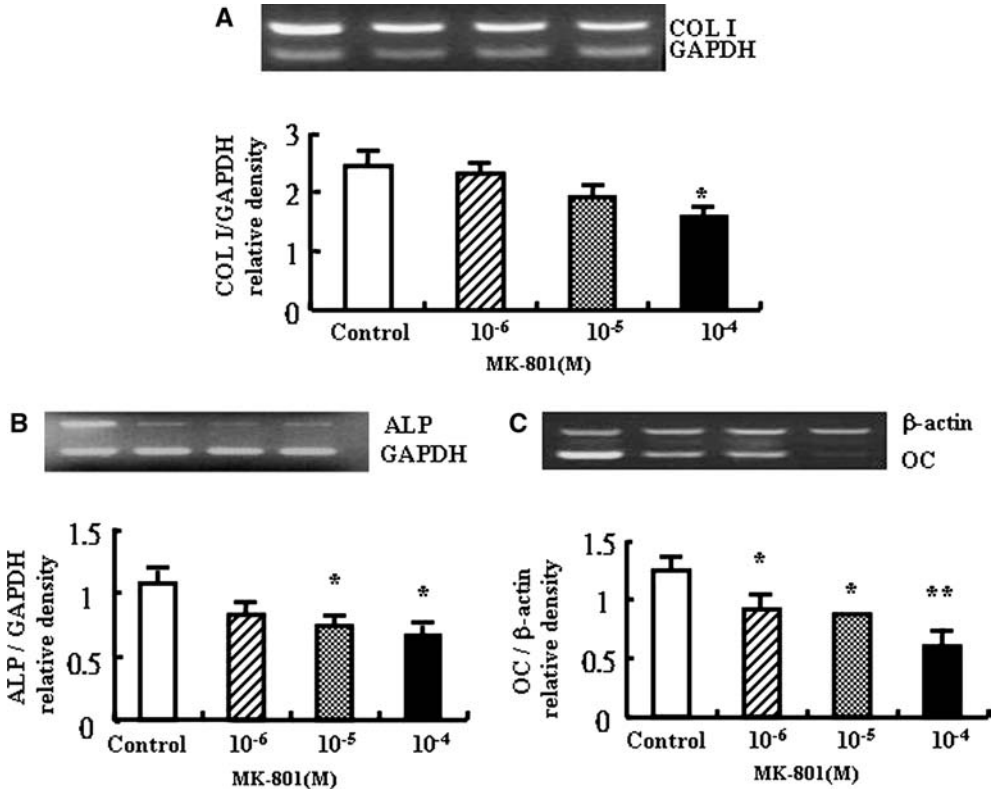

Fig. 6 NMDA receptor blockade down-regulated the expressions of osteogenic genes in cultured osteoblasts. Representative PT-PCR images of (**A**) collagen type I (*COL I*), (**B**) alkaline phosphatase (*ALP*) and osteocalcin (*OC*), and housekeeping genes, β-actin and GAPDH, from fetal rat calvarial osteoblasts are shown. The ratios of the relative optical densities from the band of cbfa1/Runx2 to that of β-actin were compared among cultures treated with different concentrations of MK-801. Each *bar* represents mean ± SE of six replicated cultures. Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. * $P < 0.05$; ** $P < 0.01$ in comparison with the control group

sites [39]. Furthermore, it was indicated that NMDA enhanced AP-1 translocation to nuclei in osteoblastic cells [40]. Accordingly, mechanical loading may stimulate osteoblastic functions through NMDA signaling, AP-1 translocation and cbfa1/Runx2 expression and eventually enhances the expressions of osteogenic genes. However, this hypothesis needs direct evidence to be proven. In this study, we found that mechanical unloading suppressed the expressions of cbfa1/Runx2, collagen type I, ALP and osteocalcin, and coincidently also NMDA receptors. At this step, we cannot conclude that the down-regulation of collagen type I, ALP and osteocalcin resulted from the down-regulation of cbfa1/Runx2 through NMDA mediation or other pathways involved in the regulation of the individual osteogenic gene. Nevertheless, based on the findings of this study, it provides a foundation to further clarify the network of the gene regulation affected by mechanical loading.

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