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Original Article

Multiple signaling pathways involved in stimulation of osteoblast differentiation by *N*-methyl-*D*-aspartate receptors activation *in vitro*

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Aim: Glutamate receptors are expressed in osteoblastic cells. The present study was undertaken to investigate the mechanisms underlying the stimulation of osteoblast differentiation by *N*-methyl-*D*-aspartate (NMDA) receptor activation *in vitro*.
Methods: Primary culture of osteoblasts was prepared from SD rats. Microarray was used to detect the changes of gene expression. The effect of NMDA receptor agonist or antagonist on individual gene was examined using RT-PCR. The activity of alkaloid phosphotase (ALP) was assessed using a commercial ALP staining kit.
Results: Microarray analyses revealed that 10 genes were up-regulated by NMDA (0.5 mmol/L) and down-regulated by MK801 (100 μmol/L), while 13 genes down-regulated by NMDA (0.5 mmol/L) and up-regulated by MK801 (100 μmol/L). Pretreatment of osteoblasts with the specific PKC inhibitor Calphostin C (0.05 μmol/L), the PKA inhibitor H-89 (20 nmol/L), or the PI3K inhibitor wortmannin (100 nmol/L) blocked the ALP activity increase caused by NMDA (0.5 mmol/L). Furthermore, NMDA (0.5 mmol/L) rapidly increased PI3K phosphorylation, which could be blocked by pretreatment of wortmannin (100 nmol/L).
Conclusion: The results suggest that activation of NMDA receptors stimulates osteoblasts differentiation through PKA, PKC, and PI3K signaling pathways, which is a new role for glutamate in regulating bone remodeling.

Keywords: bone; osteoblast; glutamate receptor; NMDA receptor; PKA; PKC; PI3K

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Introduction

L-Glutamate (Glu) is accepted as an excitatory amino acid neurotransmitter in the mammalian central nervous system (CNS). Extracellular levels of glutamate are determined by the glutamate transporter[1, 2]. The diverse actions of *L*-glutamate in the CNS result from the existence of multiple glutamate receptors (GluR). These have been divided into two classes, metabotropic (mGluR) and ionotropic (iGluR), according to their molecular heterologies and differing intracellular signal transduction mechanisms. The iGluRs are further classified into *N*-methyl-*D*-aspartate (NMDA), *DL*-α-amino-3-hydroxy-5-methylisoxasole-4-propionate (AMPA), and kainite(KA) receptors. NMDA receptors are glutamate-gated ion channels characterized by a very high Ca^{2+} conductance[3].

In mammalian bone, NMDA receptors are expressed in osteoblasts and osteoclasts as revealed by RT-PCR, *in situ* hybridization, immunohistochemistry, and electrophy-siology[4, 5]. Bone cells and neurons possess similar, and in some cases identical glutamate signaling machinery and receptors[5].

Bone loss is associated with a reduction in nerve endings that immunostain for glutamate. Glutamate-containing neuronal endings have been described in a dense and intimate network in bone tissue[6]. Chenu *et al* reported that bone loss was induced in ovarectomized (OVX) rats with a reduction in neuronal density and concluded there was a functional link between the nervous system and bone loss after ovariectomy[7]. Hinoi *et al* reported that administration of glutamate significantly prevented the decreased bone mineral density in both the femur and the tibia in ovariectomized mice *in vivo*[8]. All these findings suggest that the neurotransmitter glutamate may play a role in bone remodeling.

NMDA has been shown to promote the proliferation and

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differentiation of hippocampal neural progenitor cells (NPCs) *in vitro* by activating NMDA receptors[9]. NMDA is mitogenic for MC3T3-E1 osteoblastic cells and glutamate has been reported to promote the viability of primary human osteoblasts *in vitro*[10]. Blockade of NMDA receptors in rat primary osteoblasts inhibits expression of markers of bone formation *in vitro*[4, 11]. Previously, we demonstrated that activation of NMDA receptors promoted rat primary osteoblast differentiation and that one of the possible mechanism was ERK1/2 activation[12].

In the central nervous system (CNS), activation of the Ca^{2+}-permeable NMDA receptors results in an increase in Ca^{2+} influx. The Ca^{2+} signals then activate several Ca^{2+}-dependent kinases[13]. Kinases which have been suggested to be important include protein kinases C and A (PKC and PKA), and PI3 kinase (PI3K). PKC is localized at excitatory synapses containing NMDA receptors and might be involved in NMDA-evoked ERK1/2 phosphorylation[14]. In hippocampal neurons NMDA played a neuroprotective role through the PKA signaling pathway[15], and in striatal and cortical neurons, PI3K is critically involved in NMDA receptor activation[16–18]. Thus, the above signaling pathways may positively respond to signals from ionotropic types of glutamate receptors in the CNS.

Locally elevated extracellular Ca^{2+} levels have been suggested to play a role in regulating bone remodeling. Signaling pathways such as phospholipase C (PLC), PKC, mitogen-activated protein kinase (MAPK), and PI3K have been implicated in the modification of cellular function by Ca^{2+}[19, 20]. Since these signaling pathways are involved in NMDA receptor regulation in the CNS and were also suggested to be important by our initial microarray analysis, we hypothesized that they are also involved in NMDA-induced bone remodeling. The aim of this study was to investigate the mechanism of the effects of NMDA and MK801 (the noncompetitive antagonist of NMDA receptors) on differentiation of rat primary osteoblasts.

Materials and methods

Primary cultures of osteoblasts

Osteoblasts were prepared from calvaria of 1-day-old Sprague-Dawley rats by a sequential enzymatic digestion method as described previously[21]. The bones were cut into chips and washed with calcium- and magnesium-free phosphate-buffered saline (PBS). Calvaria were incubated at 37 °C for 20 min with 0.25% trypsin and 1 h with 0.1% type II collagenase in PBS with gentle mixing. Incubation with type II collagenase was then repeated. Cells released from the bone chips were collected in α-modified minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin (Gibco) and 100 μg/mL streptomycin (Gibco), followed by centrifugation at 1000 revolutions per minute for 10 min. The resulting pellets were re-suspended in α-MEM containing 10% FBS. Cells were plated at appropriate density, and cultured at 37 °C under 5% CO_2. Culture medium was changed every 2 d.

Rat osteoblasts ($1.5\times10^5/cm^2$) were plated in 6-well plates in α-MEM containing 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL ascorbic acid (Sigma) and 5 mmol/L sodium β-glycerophosphate (Sigma) for measurement of differentiation markers. After 4 d of culture, cells were starved with serum-free α-MEM and 0.2% bovine serum albumin (BSA) for 12 h. Cultures were also exposed to fresh serum-free medium with 0.2% BSA with or without NMDA, MK801 (both from Tocris Cookson Ltd UK), or other inhibitors.

RNA, cDNA preparation, and quantitative real-time PCR

To ensure statistical significance of microarray analyses, cultures of osteoblasts were incubated in osteogenic medium with 0.2% BSA containing NMDA or MK801 for 48 h. Total RNA was isolated from primary rat calvarial cells with TRIzol reagent (Invitrogen). RNA (1 μg) was reverse transcribed to cDNA with the Invitrogen Superscript Kit. Corresponding probes were hybridized to Illumina GeneChip arrays and subjected to bioinformatics analyses. cDNA was amplified with the Takara SYBR Green RT-PCR kit using gene-specific primers (Table 1) in the Real-Time PCR Detection System (Roche). Quantification and normalization to GAPDH amplicons was performed. Statistical analyses were performed with Prism 5.0 (GraphPad Software, San Diego, CA).

As might be expected, many genes that have previously been demonstrated to be NMDA or MK801-regulated can be found in our lists of genes as commonly regulated in the treatment regimens (Table 2 or 3). This implies that the microarray results and analyses are reliable. To validate this further, we used quantitative real time RT-PCR to examine the effect of NMDA or MK801 on individual genes. We examined the following ten genes (*Nov, Stc1, Anxa1, Tspan8, Dab2, Nfkb2, Mmp12, Fmo1, Colec12, and Fap*) which were commonly up-regulated by NMDA and down-regulated by MK801 as shown in Table 2. We also examined thirteen genes (*Cib1, Ddit3, Gaa, Herpud1, Ninj1, Trappc2, Rpo1-2, Slc20a1, Slc3a2, Trib3, Yars, Serpinb2, and Thop1*) which were commonly down-regulated by NMDA and up-regulated by MK801 as shown in Table 3.

Gene ontology analysis

http://omicslab.genetics.ac.cn/GOEAST/php/illumina.php was applied to GO analysis. We chose only GO categories that had a *P*-value of <0.001 and a log odds-ratio of >1.5-fold.

Alkaline phosphatase synthesis

Rat calvarial osteoblasts were plated at a density of 1.5×10^5/well into 6-well culture plates. After treatments, cells were washed three times with ice-cold PBS, scraped into 0.5 mL of ice-cold 0.5% Triton X-100 solution and lysed by ultrasonication in an ice bath for 2 min. The supernatant was centrifuged at 14 000×*g* and 4 °C for 5 min, then stored frozen at -20 °C until measurement of alkaline phosphatase levels. For the determination of these levels, cell lysates were incubated in a 96-well plate with 100 μL of 0.1 mol/L $NaHCO_3$-Na_2CO_3 buffer (pH 10.0) containing 0.5% Triton X-100, with *p*-nitrophenylphosphate as substrate for 30 min at 37 °C. The absorbance

Table 1. Primer sequences used for PCRs.

Gene	Primer sequence (Forward/Reverse)	Product length (bp)	Reference
Cib1	AGTCGCCTGTCTAAGGAGCT (Forward) ATCTCCTGCTTGGTCAGGAA (Reverse)	85	NM_031145.1
Ddit3	AGGTCCTGTCCTCAGATGAAA (Forward) TAGGGATGCAGGGTCAAGAGT (Reverse)	116	NM_024134.2
Gaa	TACCGACCTCACCATCAGGAA (Forward) CTCCTGGGTGATGCCTTTGT (Reverse)	166	NM_199118.1
Herpud1	AAAAGCCAGAAGCCAGCACAA (Forward) TTGAGGAATCCCCAGGATACT (Reverse)	107	NM_053523.1
Ninj1	TCGTGGTGCTCATCTCCATCT (Forward) ATGAAAACCAGCCCCGTG (Reverse)	145	NM_012867.1
Nfkb2	ATCTGGGTGTCCTGCATGTAA (Forward) CCGTACAATGCTCAAATCCA (Reverse)	177	NM_001008349.1
Rpo1-2	ACCATCTGCAAAGAGCTCAA (Forward) TGATGGGAACATGCCAAGAA (Reverse)	166	NM_031773.1
Slc20a1	GGCAACTAGTGGCTTCGTTTT (Forward) AGACGAACCAAGACATCACAA (Reverse)	165	NM_031148.1
Slc3a2	AGAGATGGTGGCACAAGGG (Forward) TCTGGTTCTTGTGAATTGGG (Reverse)	174	NM_019283.1
Trib3	TTTTCCGACAGATGGCTAGTG (Forward) TTGTCCCACAGAGAGTCGTCT (Reverse)	189	NM_144755.2
Yars	CGTGGCCTACTTTGTACCCAT (Forward) TGCATGAAGGTCTGCAAACA (Reverse)	96	NM_001025696.1
Serpinb2	CACTCCAGGAAACCCAGAGAA (Forward) TTTGTCTCTTGCTTGTGCCT (Reverse)	129	NM_021696.1
Thop1	GGCTGACAAGAAGCTCTCAGA (Forward) TCTTTCGGGATTTTCTCCTG (Reverse)	110	NM_172075.2
Nov	TTCCTGCTCCTCCATCTCTTA (Forward) TCGGTGATATACTGGGGCA (Reverse)	115	NM_030868.2
Stc1	TGCTAAATTTGACACTCAGGG (Forward) AATGGCAAGGAAGACCTTGGA (Reverse)	106	NM_031123.2
Anxa1	ATGTTGCTGCCTTGCACAAA (Forward) TGAGCATTGGTTCTCTTGGT (Reverse)	97	NM_012904.1
Tspan8	TGGGTTTCCTGGGATGCT (Forward) ATACCTGCGGCTACTTGCAGA (Reverse)	126	NM_133526.1
Dab2	TTTGGTTATGTGTGTGGAGGA (Forward) TTCAGCCTGTTGCCCTGTTT (Reverse)	88	NM_024159.1
Mmp12	ATGAAGCGTGCGGATGTAGA (Forward) CTCCATGATCTCCAAATGCAA (Reverse)	147	NM_053963.1
Fmo1	ATGCAACCCAGTTCAACCTT (Forward) ATTGTCCAGAGACAGCGAAA (Reverse)	118	NM_012792.1
Trappc2	TGTAATTGTTGGCCACCATGA (Forward) TGACGGTGATCATCTTTGGA (Reverse)	113	NM_001024965.1
Colec12	CAGGTCCCTCAGGAGCAAT (Forward) CAAAAATTTCTTTCTCCACCG (Reverse)	159	NM_001025721.1
Fap	TTGAAGGTTACCCTGGAAGAA (Forward) TGGCTTTGTAGCTGAAACTTG (Reverse)	157	NM_138850.1
GAPDH	CTCAACTACATGGTCTACATGT (Forward) CTTCCCATTCTCAGCCTTGACT (Reverse)	132	NM_017008.3

of *p*-nitrophenol liberated in the reactive solution was read at 405 nm. Diluted cell lysates were measured at 740 nm for total protein content using the BCA method (Bio-Rad Protein Assay kit, Bio-Rad Laboratories, Richmond, CA, USA). ALP activity in the cells was normalized for total protein content of the cell lysate.

Western blotting

Cells treated with 0.5 mmol/L NMDA and wortmannin (100 nmol/L) (Cell Signaling, USA) for the indicated times were lysed and the protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). For Western blotting, 50 mg of total cell lysates was subjected

Table 2. The common genes up-regulated by NMDA and down-regulated by MK801 based on GO analysis.

Name	Symbol	Accession
Nephroblastoma overexpressed gene (Nov)	Nov	NM_030868.2
Stanniocalcin 1 (Stc1)	Stc1	NM_031123.2
Annexin A1 (Anxa1)	Anxa1	NM_012904.1
Tetraspanin 8 (Tspan8)	Tspan8	NM_133526.1
Disabled homolog 2 (*Drosophila*) (Dab2)	Dab2	NM_024159.1
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	Nfkb2	NM_001008349.1
Matrix metallopeptidase 12 (Mmp12)	Mmp12	NM_053963.1
Flavin containing monooxygenase 1 (Fmo1)	Fmo1	NM_012792.1
Collectin sub-family member 12 (Colec12)	Colec12	NM_001025721.1
Fibroblast activation protein (Fap)	Fap	NM_138850.1

Table 3. The common genes down-regulated by NMDA and up-regulated by MK801 based on GO analysis.

Name	Symbol	Accession
Calcium and integrin binding 1 (calmyrin)	Cib1	NM_031145.1
DNA-damage inducible transcript 3	Ddit3	NM_024134.1
Glucosidase, alpha, acid	Gaa	NM_199118.1
Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	Herpud1	NM_053523.1
Ninjurin 1	Ninj1	NM_012867.1
Similar to RIKEN cDNA 0610009B22 (RGD1306925)	Trappc2	NM_001024965.1
RNA polymerase 1-2	Rpo1-2	NM_031773.1
Solute carrier family 20 (phosphate transporter), member 1	Slc20a1	NM_031148.1
Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	Slc3a2	NM_019283.1
Tribbles homolog 3 (Drosophila)	Trib3	NM_144755.1
Tyrosyl-tRNA synthetase	Yars	NM_001025696.1
Serine (or cysteine) proteinase inhibitor, clade B, member 2	Serpinb2	NM_021696.1
Thimet oligopeptidase 1	Thop1	NM_172075.1

to SDS-polyacrylamide gel electrophoresis. The protein was transferred to polyvinylidene difluoride membranes using transfer buffer (50 mmol/L Tris, 190 mmol/L glycin, and 10% methanol) at 120 V for 2 h. The membranes were incubated with blocking buffer (50 mmol/L Tris, 200 mmol/L NaCl, 0.2% Tween 20, and 3% bovine serum albumin) overnight at 4 °C. After washing three times with washing buffer (blocking buffer without 3% bovine serum albumin) for 10 min each, the blot was incubated with primary antibody (PI3K, phosphorylated PI3K) for 12 h, followed by horseradish peroxidaselabeled secondary antibody for 1 h. The membranes were washed again, and detection was performed using an ECL kit (ECL Plus Western Blotting Detection System, GE Healthcare UK Limited, Little Chalfont, UK) and exposed to film.

Statistical analysis

Each experiment was performed with at least three independent samples. The results are expressed as the mean±SD, unless otherwise indicated. Statistical significance of the observed differences was analyzed by one-way ANOVA where appropriate. A P value <0.05 was considered statistically significant.

Results

cDNA microarray and gene ontology analysis

Genes whose expression was changed in opposite directions by NMDA and MK801 treatment were defined as being commonly up-regulated or down-regulated genes. According to the ratio values, 353 genes were up-regulated by NMDA, 106 genes down-regulated by MK801 and hence 83 genes were the commonly up-regulated genes. There were 50 genes down-regulated by NMDA, 297 genes up-regulated by MK801 and 27 were the commonly down-regulated genes (Figure 1). We then examined the significant GO categories and genes by http://omicslab.genetics.ac.cn/GOEAST/php/illumina.php. A P-value of <0.001 and a log odds-ratio >1.5-fold were selected as the significant criteria. This narrowed the numbers of genes to 10 in the group of common genes that were up-regulated by NMDA and down-regulated by MK801 (Table 2), and 13 in the group of common genes down-regulated by NMDA and up-regulated by MK801 (Figure 2, Table 3).

Effects of inhibiting PKC, PKA, and PI3K on indexes of osteoblast differentiation

From the results of the microarray analysis as well as some previously published work in other cell types, we noted several genes (*Anxa1, Mmp12, Stc1, Trib3, NF-κB, Ddit3*) involved in PKC, PKA, and PI3K signaling pathways (see discussion). To determine the involvement of PKC in NMDA-mediated osteoblast differentiation, serum-starved cells were pretreated with Calphostin C (0.05 μmol/L), a specific inhibitor of PKC, for 90 min followed by coincubation with 0.5 mmol/L NMDA for 48 h. Cell morphology was assessed using a commercial ALP staining kit. Basal levels of ALP activity were unaffected by treatment of osteoblastic cells with Calphostin C alone. However, this treatment did lead to an observable reduction in alkaline phosphatase levels (Figure 3).

To determine whether activation of PKA is involved in NMDA-induced osteoblast differentiation, cells were pretreated with the PKA inhibitor H-89 (20 nmol/L). After incubation in serum-free medium for 12 h, cells were pretreated with 20 nmol/L H-89 for 90 min, followed by coincubation with 0.5 mmol/L NMDA for 48 h. Inhibition of PKA by H-89 treatment led to a decrease in NMDA-stimulated ALP activity levels (Figure 3). The ability of this inhibitor to curtail the effects of NMDA on osteoblast differentiation suggests that PKA activation is involved in NMDA-induced osteoblast differentiation.

We further explored signal transduction components related

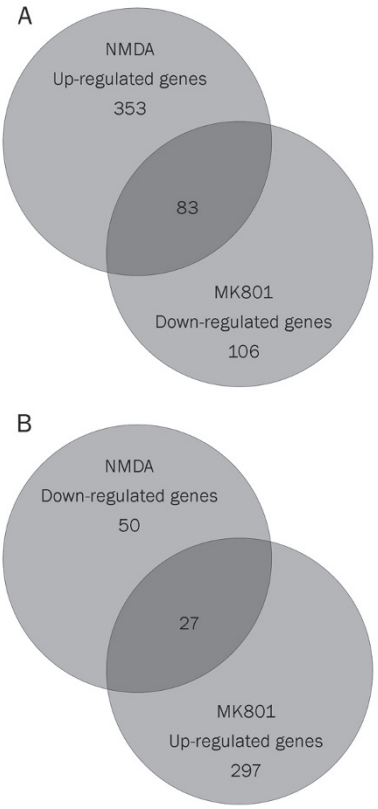

Figure 1. Number of genes changed by NMDA or MK801 treatment regimens. Genes identified by Illumina GeneChip microarray were changed with treatment by at least 1.5-fold compared to vehicle. The number of genes (both up- and down-regulated) common to both treatments is noted in the overlap of the Venn diagram.

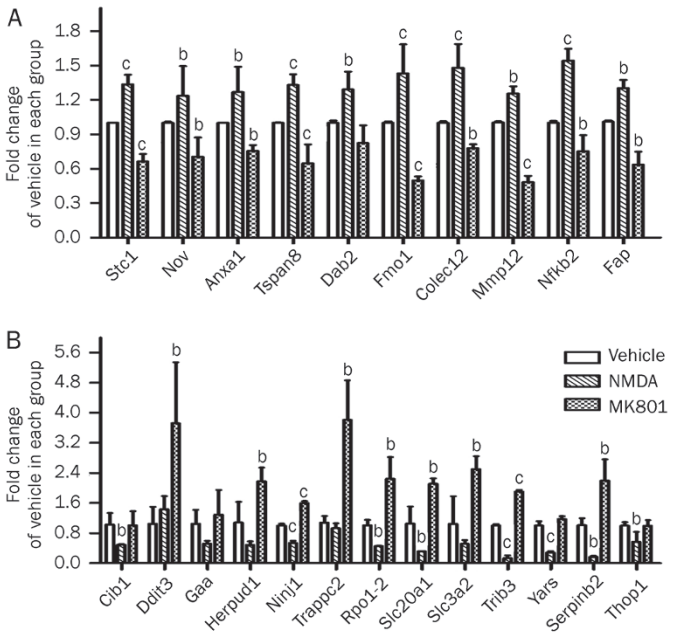

Figure 2. Verification of common genes according to GO analysis by real-time PCR. A: Genes up-regulated by NMDA and down-regulated by MK801; B: Genes down-regulated by NMDA and up-regulated by MK801. Primary calvarial osteoblasts were cultured in medium with 0.2% BSA containing 0.5 mmol/L NMDA or 100 μmol/L MK801 for 48 h. mRNAs were isolated after 48 h and subjected to quantitative real-time PCR with primers described in Table 1. Comparative threshold values represent the mean of three samples normalized to GAPDH levels. Values are relative to those obtained from the vehicle groups. $^{b}P<0.05$, $^{c}P<0.001$ by one-way ANOVA versus the vehicle groups.

to NMDA-induced osteoblast differentiation by examining the involvement of PI3K using the PI3K inhibitor wortmannin. Cells were pretreated with wortmannin (100 nmol/L) for 90 min, followed by coincubation with 0.5 mmol/L NMDA for 48 h. This treatment protocol also led to a decrease in NMDA-stimulated ALP activity levels (Figure 3). The ability of these three inhibitors to curtail the effects of NMDA on markers of osteoblast differentiation suggests that activation of PKC, PKA, and PI3K is involved in the phenomenon of NMDA-induced osteoblast differentiation.

To assess whether PI3K were activated by NMDA, we assessed phosphorylation of PI3K using phospho-antibodies against phosphorylated peptides derived from PI3K. We found that 0.5 mmol/L NMDA induced a rapid increase in PI3K phosphorylation with maximal levels at 15 min. Prolonged NMDA stimulation up to 30 min, however, resulted in a decrease of phosphorylated PI3K levels toward baseline (Figure 4A).

To determine whether activation of PI3K is involved in NMDA-induced osteoblast differentiation, cells were pretreated with the PI3K inhibitor wortmannin. After incubation in serum-free medium for 12 h, cells were pretreated with 100 nmol/L wortmannin for 90 min, followed by coincubation with 0.5 mmol/L NMDA for 15 min. Inhibition of PI3K by wortmannin treatment led to a decrease in NMDA-stimulated phosphorylated PI3K levels (Figure 4B). The ability of this inhibitor to curtail the effects of NMDA on osteoblast differentiation suggests that PI3K activation is involved in NMDA-induced osteoblast differentiation (Figure 5).

Discussion

Our data suggest that NMDA promotes osteoblast differentiation via PKA, PKC and PI3K signaling pathways. These findings also demonstrate that NMDA directly acts on and affects osteoblasts and that the NMDA receptors expressed in primary rat calvaria osteoblasts are functional.

Within the large family of iGluRs, NMDARs constitute a subfamily identifiable by a specific molecular composition and unique pharmacological and functional properties[22, 23]. The most commonly used agonist at the glutamate recognition site of NMDA receptors is NMDA itself. However, it is not a substrate that promotes glutamate uptake[24]. Activation of NMDA receptors has been shown to be important in normal expression of bone matrix proteins[4, 12]. We have previously demonstrated that NMDA increased osteoblast ALP activity and osteocalcin (OC) in a dose-dependent manner, while the NMDA receptor antagonist MK801 reduced these effects[12].

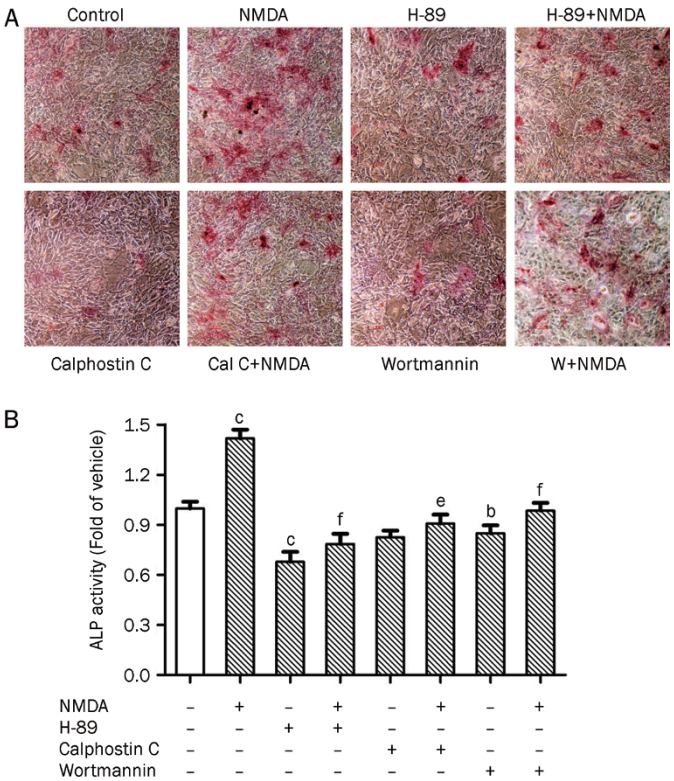

Figure 3. Effect of inhibitors of signal transduction on alkaline phosphatase levels. Cells were pretreated with vehicle or the PKA inhibitor H-89 (20 nmol/L), the PKC inhibitor Calphostin C (Cal C, 0.05 μmol/L), or the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (W, 100 nmol/L) for 90 min. Cells were then treated with 0.5 mmol/L NMDA for 48 h. A: ALP staining study; B: ALP activity study. Mean±SD (n=8). ^{b}P<0.05, ^{c}P<0.001 *vs* control; ^{e}P<0.05, ^{f}P<0.001 *vs* the group treated with NMDA only.

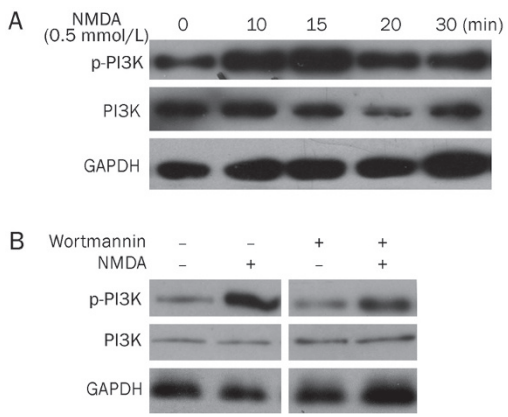

Figure 4. Effects of NMDA on PI3K activation in osteoblastic cells. Cell lysates were subjected to Western blot and incubated with PI3K or phosphorylated PI3K antibodies. (A) Cells are exposed to 0.5 mmol/L NMDA for 0, 10, 15, 20, and 30 min. PI3K was phosphorylated by NMDA and the peak reached at 15 min. (B) Cells incubated with wortmannin (100 nmol/L) for 90 min prior to treatment with 0.5 mmol/L NMDA, and then treated with or without NMDA for 15 min. The suppression of phosphorylated PI3K induced by NMDA was observed in the presence of wortmannin.

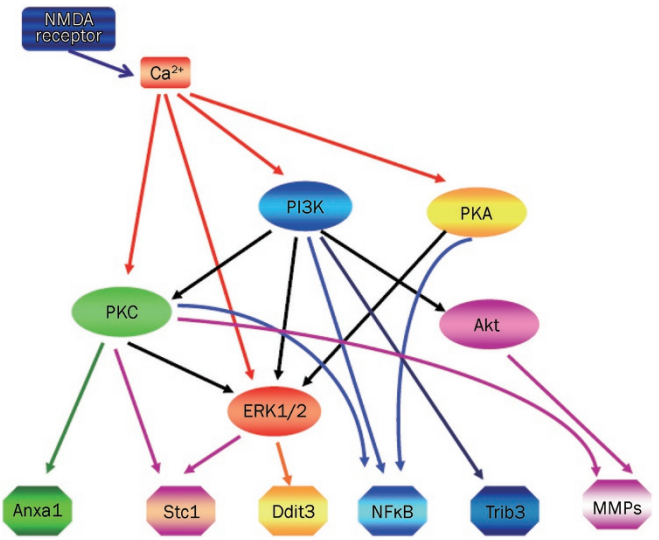

Figure 5. Schematic representations of signaling pathways from NMDA receptors to the multiple signaling system.

We have also observed that NMDA increases ALP activity and OC expression in a time-dependent manner and that the peak of this increase reached 48 h after treatment (data not shown). Together, these observations suggest that NMDA regulates osteoblast differentiation via NMDA receptors.

In the present study, we employed cDNA microarray analysis, which was expected to be more sensitive than cDNA subtraction analysis, for the detection of specific genes related to the stimulation of NMDA receptors in rat osteoblasts. Annexin 1 (Anxa1) is one member of a family of phospholipid and calcium-binding proteins of which 20 are known at present[25]. It translocates from the cytoplasm to the outer cell surface via a Ca^{2+}-dependent mechanism[26–28]. It is a substrate for protein kinase C and protein tyrosine kinases and has multiple phosphorylation sites as well as calcium and phospholipid binding properties[29]. Anxa1 was also shown to be activated as a consequence of sequential PI3K-PKC activation[30, 31]. Matrix metalloproteinases (MMPs) are a family of secreted or transmembrane zinc-dependent endopeptidases. The activation of MMP3, MMP12 and MMP13 was correlated with activation of the PI3K/Akt signaling cascade in microglial cells[32]. In epithelial cells and fibroblasts, MMP12 and MMP13 could be up-regulated in a PI3K, PKCδ and ERK1/2-dependent manner[33]. The stanniocalcins, comprising Stc1 and Stc2, are secreted homodimeric glycoprotein hormones with little homology to other proteins[34]. Stc1 was originally identified as a hormone secreted by the corpuscles of the stannius inteleost fish[35] and in fish appears to function primarily in the prevention of hypercalcaemia mediated via the ability of Stc1 to reduce calcium uptake by gills and inhibit intestinal calcium transport in the gut[36–38]. In human endothelial cells Stc1 mRNA expression was up-regulated primarily through PKC, ERK and Ca^{2+} signaling pathways[39]. Trib3 (tribbles 3) is a mammalian homologue of *Drosophila tribbles*[40–42]. Expression of Trib3 was found to be PI3K-dependent in prostate

cancer cells[43]. Furthermore, it has been suggested that the tribbles protein family is involved in regulation of the MAP kinase pathway[44]. Nuclear factor-κB (NF-κB) is a ubiquitous heterodimeric transcription factor that regulates inflammation and cell survival and differentiation[45, 46]. Many different kinases, including PKA, PKC, GSK-3β, PI3K, AKT, p38, NIK and even IKK, have been shown to induce phosphorylation of NF-κB directly or indirectly[45–47]. Regulation of NF-κB has also previously been suggested to depend on the PLCβ/PKC pathway[48, 49]. The PI3K/Akt signaling pathway seems able to positively regulate NF-κB activity[50, 51]. Studies have demonstrated that different stress response pathways mediating Ddit3 (DNA-damage inducible transcript 3) expression are regulated by protein kinases[52, 53]. Ddit3 is reported to be induced during serum starvation, glutamine deprivation, growth to confluency and upon exposure to tunicamycin, which promotes accumulation of proteins in the ER by preventing glycosylation[54]. Ddit3 protein was increased in pancreatic β-cells grown in high glucose that were exposed to U0126 to block ERK1/2 activity[55]. Our data showed Anxa1, MMP12, Stc1 and NF-κB were all up-regulated by NMDA in osteoblasts, while Trib3 and Ddit3 were down-regulated. In addition, it suggests that ERK, PKA, PKC and PI3K signaling pathways may be involved with changes of these genes' expression levels in NMDA-treated osteoblasts.

In the CNS, activation of NMDA receptors increases calcium influx, which is associated with spatial long term memory in the Morris water maze[56] through the PKA signaling pathway[56–58]. Repetitive stimulation of glutamatergic NMDA receptors result in PKA and PKC activation *in vivo*[59]. The high Ca^{2+} permeability of NMDA receptor channels in bone is a characteristic similar to that of receptors expressed in the CNS[5, 60]. Ca^{2+} flux could trigger a number of second messenger responses that could ultimately be responsible for the anabolic effects of NMDA stimulation in bone. The MAPK, PKC, PKA and PI3K signaling pathways can all be directly activated downstream of calcium influx[61]. The activation of protein kinase C (PKC) and/or protein kinase A (PKA) plays an essential role in osteoblast differentiation[62]. In our previous study, we have observed activation of the ERK1/2 signaling pathway when osteoblasts were treated with NMDA[12]. With this background information, we further pursued the results of our microarray analysis information and specifically studied the PKC, PKA and PI3K signaling pathways to see if these signaling pathways are involved in NMDA-mediated osteoblast activation, independent of ERK1/2. We found that cells staining for ALP were significantly decreased by treatment with the PKA inhibitor H-89 or the PKC inhibitor Calphostin C. However, the ALP staining cells recovered after treatment with NMDA, which suggests a mechanism for NMDA-induced osteoblast differentiation that is, at least partly, dependent on PKA and PKC signaling. NMDA increased PI3K phosphorylation which is kinetically correlated to concomitant ERK1/2 phosphorylation in rat striatal neurons[63]. It was reported that intracellular Ca^{2+}- regulated pathways signal through PI3K in osteoblasts subjected to mechanical strain[64]. The ability of the chemical inhibitor of PI3K, wortmannin, to significantly reduce NMDA-induced expression of the osteoblast differentiation marker ALP suggests that PI3K is involved in NMDA-mediated osteoblastic cell differentiation.

In summary, we have demonstrated that activation of NMDA receptors promotes osteoblast differentiation via PKA, PKC and PI3K signaling mechanisms which are independent of ERK1/2 signaling. The screening for specific genes carried out in this work allowed the discovery of signaling pathways which are associated with these genes. These findings yield new avenues by which to look for therapeutic targets in osteoporosis and provide useful direction for further investigation of the mechanisms involved.

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Author contribution

Dr Jie-li LI designed the project, performed the research and wrote the paper; Dr Lin ZHAO worked on the data for revision; Dr Bin CUI helped on detailed research work; Dr Lian-fu DENG and Dr Guang NING contributed overview advice; Dr Jian-min LIU provided fundings on all the work and designed the project.

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