J Pharmacol Sci **106**, 536 – 541 (2008)

Journal of Pharmacological Sciences

Forum Minireview

Pharmacological Topics of Bone Metabolism: Glutamate as a Signal Mediator in Bone

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Received November 24, 2007; Accepted January 9, 2008

Abstract. The view that L-glutamate (Glu) is an excitatory amino acid neurotransmitter in the mammalian central nervous system is prevailing on the basis of successful cloning of a number of genes encoding different signaling molecules, such as Glu receptors for the signal input, Glu transporters for the signal termination and vesicular Glu transporters for the signal output through exocytotic release. Little attention has been paid to an extracellular transmitter role of Glu in peripheral neuronal and non-neuronal tissues, by contrast, whereas recent molecular biological and pharmacological analyses including ours give rise to a novel function for Glu as an autocrine and/or paracrine signal mediator in bone comprised of osteoblasts, osteoclasts and osteocytes, in addition to other peripheral tissues including pancreas, adrenal and pituitary glands. Emerging evidence suggests that Glu could play a dual role in mechanisms underlying the maintenance of cellular homeostasis as an excitatory neurotransmitter in the central nervous system and as an extracellular signal mediator in peripheral autocrine and/or paracrine tissues. In this review, therefore, we would outline the possible signaling system for Glu to play a role as an extracellular signal mediator in mechanisms underlying maintenance of the cellular homeostasis in bone.

Keywords: glutamate, osteoblast, osteoclast, NMDA receptor, cystine/glutamate antiporter, bone metabolism

Introduction

L-Glutamate (Glu) is believed to play a dual role as an excitatory amino acid neurotransmitter and as an excitotoxin in the mammalian central nervous system (CNS). These actions are mediated through particular Glu receptors (GluRs) categorized into two major groups (1, 2). One is ionotropic Glu-gated ion channels (iGluRs) that are further classified into DL-*α*-amino-3-hydroxy-5-methylisoxasole-4-propionate (AMPA), kainate (KA), and *N*-methyl-D-aspartate (NMDA) subtypes (3, 4), whereas the other is G-protein-coupled metabotropic receptors (mGluRs) classified into the three functional groups, group I (mGluR1 and mGluR5), group II (mGluR2 andmGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8) subtypes (5, 6). Excitatory amino acid transporters (EAATs) are required for the termination of signal transduction mediated by Glu as well as for the prevention of neurotoxicity mediated by this endogenous excitotoxin in the CNS. These transporters are classified into 5 different subtypes, including Glu aspartate transporter (GLAST) (EAAT1), Glu transporter-1 (GLT-1) (EAAT2), excitatory amino acid carrier (EAAC1) (EAAT3), EAAT4, and EAAT5 to date (7, 8). In addition to these Glu transport systems mentioned above, sodium-independent, chloride-dependent high affinity Glu uptake system termed as the cystine/Glu antiporter has been identified in many central and peripheral tissues (9, 10). This antiporter is a heterodimeric complex between the CD98 heavy chain, also referred to as 4F2hc, ubiquitously present in various tissues and the xCT light chain responsible for determination of the substrate specificity. In addition, the third Glu transport system, which is sodium-dependent with cystine, Glu, and aspartate as

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Published online in J-STAGE
doi: 10.1254/jphs.FM0070243

substrates, has also been found in rat alveolar type 2 cells and in astrocytes (11, 12). Moreover, vesicular Glu transporters (VGLUTs) are essential for the signal output through the condensation of Glu into vesicular constituents for subsequent exocytotic release upon stimulation. Within the CNS, both VGLUT1 (13) and VGLUT2 (14) isoforms are supposed to suffice for the definition of an excitatory neuronal phenotype, while VGLUT3 is expressed in a number of cells shown to release Glu through exocytosis including dopaminergic, GABAergic, and serotonergic neurons as well as astrocytes (15).

On the other hand, two distinct cell types are known to sophisticatedly regulate bone formation and remodeling in bone tissues (16, 17). These are bone-forming osteoblasts and bone-resorbing osteoclasts. The osteoblast lineage is derived from primitive multipotent mesenchymal stem cells with potentiality to differentiate into bone marrow stromal cells, chondrocytes, muscles, and adipocytes (18), while osteoclasts are multinucleated cells (MNCs) derived from hematopoietic stem cells shared with macrophage and dendritic cell lineages (19, 20). Osteoclastogenesis is a multi-step process dependent on the intimate cellular interaction of myeloid pre-osteoclastic precursors with either osteoblasts or stromal cells under the influence by a wide range of local autocrine and/or paracrine factors such as macrophage-colony stimulating factor (M-CSF) (21, 22) and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) (23, 24). In addition to these extracellular factors, recent studies have raised the possibility that Glu may be one of the endogenous factors used for intercellular communications in bone through the activation of NMDA receptors expressed by bone-resorbing osteoclasts (25 – 27) as seen in bone-forming osteoblasts (28 – 31). For example, the addition of an NMDA-receptor antagonist inhibits cell differentiation and bone-resorbing activities in cultured osteoclasts expressing both NR1 and NR2 subunits required for the heteromeric assembly to functional cationic channels (25 – 27). In these previous studies using bone marrow stromal cells toward the growth of osteoclasts, however, the possibility that functional expression of glutamatergic signaling molecules may be at least in part derived from osteoblasts contaminated in cultured preparations is not ruled out.

In the present review, therefore, we have focused on the evaluation of the possible expression and functionality of a variety of different glutamatergic signaling machineries in primary cultured mouse osteoclasts defective of the contamination with either osteoblasts or stromal cells through the usage of recombinant mouse RANKL and M-CSF, which are both key extracellular regulators produced and released by osteoblasts and osteogenic stromal cells, for differentiation of hematopoietic bone marrow precursors prepared after Ficoll gradient centrifugation.

Isolation of osteoclasts

We have at first attempted to establish accurate and reproducible isolation procedures for osteoclasts devoid of osteoblasts and stromal cells. Osteoclasts are multinucleated cells derived from the fusion of mononuclear hematopoietic precursors, but their differentiation is dependent on the presence of two essential factors that are usually provided by cells of an osteoblastic lineage. Osteoblasts or bone marrow stromal osteoblast precursors secrete M-CSF and express the cell-surface protein RANKL, which binds to its receptor (RANK) expressed on cellular surfaces of osteoclast progenitors. Osteoblasts also express the soluble protein osteoprotegerin that acts as a decoy receptor for RANKL for inhibition of the osteoclast differentiation. Osteotropic agents such as PTH can promote osteoclast differentiation by elevating RANKL expression in osteoblasts. Osteoblastic RANKL expression also regulates the maturity and viability of osteoclasts in addition to regulating osteoclast differentiation.

Bone marrows were prepared from tibia and femur of adult male mice and cultured for 24 h with M-CSF in αMEM containing 10% FBS as described elsewhere in detail (32). After culturing for 24 h in the presence of M-CSF alone, supernatants were collected by gentle aspiration, followed by the lamination of non-adherent cells in the supernatants on a Ficoll gradient, and subsequent centrifugation. Cells fractionated in the monocyte fraction were defined as pre-osteoclasts and collected for suspension in MEM containing 10% FBS, M-CSF and RANKL. Cells were then cultured in MEM containing 10% FBS, M-CSF, and RANKL at 37°C under 5% CO_2.

Semi-quantitative RT-PCR analysis revealed that mRNA expression was drastically increased for all osteoclastic marker genes examined in proportion to culture periods from 1 to 6 days. These included RANK, carbonic anhydrase II, matrix metalloproteinase-9, c-fms, cathepsin K, calcitonin receptor and c-src, in addition to tartrate-resistant acid phosphatase (TRAP). To evaluate the possible contamination with osteoblasts and/or stromal cells in these mouse cultured osteoclasts differentiated from bone marrow precursors in the presence of both M-CSF and RANKL, moreover, mRNA was extracted from these primary cultured osteoclasts for subsequent RT-PCR using specific primers for type I collagen and osteocalcin, which are both known as an osteoblastic marker. In pre-osteoclasts not exposed to

RANKL and mature osteoclasts cultured for 5 days in the presence of both M-CSF and RANKL, mRNA expression was markedly found for GAPDH, but not for either type I collagen or osteocalcin. Accordingly, we have decided to use cells isolated, prepared, and cultured under adequate conditions as mouse primary cultured osteoclasts before and after the differentiation from bone marrow hematopoietic precursors.

Glutamate signaling molecules in osteoclasts

In order to analyze the expression of Glu signaling machineries including GluRs and EAATs, mRNA was extracted from cultured primary osteoclasts for subsequent RT-PCR using specific primers for each molecule. Mouse whole brain exhibited marked expression of mRNA for all Glu signaling machineries examined. These included NR1, NR2A, NR2B, NR2C, and NR2D subunits of NMDA receptors; GluR1, GluR2, GluR3, and GluR4 subunits of AMPA receptors; GluR5, GluR6, GluR7, KA1, and KA2 subunits of KA receptors; mGluR1, mGluR2, mGluR3, mGluR4, mGluR5, mGluR6, mGluR7, and mGluR8 isoforms of mGluRs; and GLAST, GLT-1, EAAC1, EAAT4, and EAAT5 isoforms of EAATs, VGLUT1, and VGLUT2 isoforms of VGLUT. In pre-osteoclasts cultured for 1 day in the presence of M-CSF alone after the isolation and mature osteoclasts cultured for 5 days in the presence of both M-CSF and RANKL, however, no mRNA expression was found for all GluRs and VGLUTs examined. In contrast to GluRs and VGLUTs, expression was seen with mRNA for GLT-1 and EAAT4, but not for GLAST, EAAC1, and EAAT5, isoforms of EAATs in primary osteoclasts before and after the differentiation from bone barrow precursors. Sequencing analysis on these amplified PCR products clearly confirmed the expression of mRNA for the corresponding Glu signaling machineries. In matured osteoclasts cultured for 5 days, moreover, high immunoreactivity was detected for EAAT4, but not for GLT-1, isoform on Western blotting.

Cystine/glutamate antiporter in osteoclasts

To analyze the expression of mRNA for the cystine /Glu antiporter requited for the biosynthesis of the intracellular antioxidant reduced glutathione (GSH) (Fig. 1), RT-PCR was conducted for both xCT and 4F2hc subunits essential for the functional heteromeric assembly to the antiporter in primary osteoclasts. Although xCT mRNA was seen in pre-osteoclasts with a dramatic decrease in matured cells cultured for 5 days, constitutive expression of mRNA was found for the 4F2hc subunit in both pre-osteoclasts and matured osteoclasts. To further examine the expression of the xCT subunit, pre-osteoclasts were cultured in either the presence or absence of RANKL for an additional 24 h, followed by the determination of xCT expression at both mRNA and protein levels by RT-PCR and immunoblotting techniques. Exposure to RANKL for 24 h markedly decreased xCT expression at both mRNA and protein levels, without affecting 4F2hc mRNA expression, as seen in matured osteoclasts.

In order to evaluate the possible effect of Glu on osteoclast differentiation, pre-osteoclasts were cultured in MEM containing both M-CSF and RANKL in either the presence or absence of Glu at concentrations of below 1 mM for 5 consecutive days. Sustained exposure to Glu at 500 μM markedly inhibited the formation of TRAP-positive MNCs with a pavement-shape, while quantitative calculation revealed that Glu significantly decreased the number of TRAP-positive MNCs in a concentration-dependent manner at concentrations of 1 μM to 1 mM. In contrast, no significant changes were seen in the number of TRAP-positive MNCs in cells cultured for 5 days with different iGluR agonists such as AMPA, KA, and NMDA, or mGluR agonists including DHPG, DCG-IV, and L-AP4, at 100 μM. Pre-osteoclasts were exposed to 500 μM Glu at different days from 0 to 5 days, followed by TRAP staining at 5 days. The number of TRAP-positive MNCs was significantly decreased following the sustained exposure to Glu for 3 to 5 consecutive days, whereas Glu did not significantly affect the number of TRAP-positive MNCs at 500 μM when exposed after 3 days until the day of TRAP staining. However, no significant alternation was found in the cellular viability in pre-osteoclasts cultured in MEM containing both M-CSF and RANKL in the presence of Glu at concentrations of over 500 μM for 5 consecutive days. In cells exposed to Glu at 0.5 to 1 mM for 5 days, moreover, a significant decrease was seen in intracellular GSH levels.

To assess the role of the cystine/Glu antiporter in osteoclast differentiation, pre-osteoclastic RAW264.7 cells were stably transfected with the expression vector containing the full-length coding regions of xCT and 4F2hc subunits or with empty vector. Expression levels of both xCT and 4F2hc subunits were examined by semi-quantitative RT-PCR, immunoblotting, and uptake analyses. Several clones of cells transfected with xCT and 4F2hc subunits showed markedly elevated expression of xCT and 4F2hc subunits compared to cells transfected with empty vector alone. In addition to the upregulation of xCT and 4F2hc expression, stable overexpression of both subunits more than tripled the activity to incorporate [^{3}H]Glu and [^{14}C]cystine in

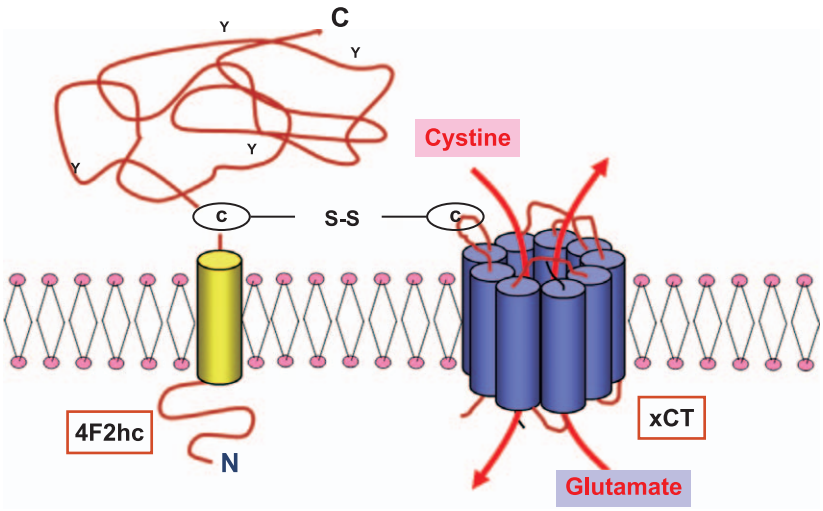

Fig. 1. Cystine/glutamate antiporter. The antiporter is functionally orchestrated by the heteromeric assembly between xCT and 4F2hc subunits. Anterograde movement is toward the incorporation of extracellular cystine into the cytoplasmic location for the biosynthesis of glutathione in exchange for intracellular glutamate, whereas in the presence of extracellular glutamate at high concentrations, intracellular cystine is exported through the retrograde operation of the antiporter to lead to the reduction of intracellular glutathione.

RAW264.7 cells. To investigate whether xCT and 4F2hc indeed affect osteoclast differentiation, cells were cultured for 4 days with RANKL in either the presence or absence of Glu. The number of TRAP-positive MNCs was similarly increased in proportion to the culture duration from 1 to 4 days in both cells with empty and expression vectors. In addition, sustained exposure to Glu at a concentration of over 500 μM significantly decreased the number of TRAP-positive MNCs in a concentration-dependent manner in both types of cells. Cultured cells were exposed to 500 μM Glu at different days of culture from 0 to 4 days, followed by TRAP staining at 4 days. In cells with stable overexpression, Glu was more efficient in decreasing the number of TRAP-positive MNCs when exposed for a period longer than 1 day up to 3 days. However, no significant difference was seen in the inhibition between both cells exposed to Glu for 4 days. Therefore, stable overexpression of both xCT and 4F2hc subunits would facilitate the inhibition by Glu of osteoclastogenesis in RAW264.7 cells in vitro.

Glutamate administration in ovariectomized mice

To examine the possible functional significance of the in vitro inhibition by Glu of osteoclastogenesis, we next conducted daily intraperitoneal administration of Glu at different doses in ovariectomized mice for subsequent determination of the bone mineral density in addition to different histomorphometric parameters 1 day after the last injection. The administration of Glu at a dose of 1 g/kg did not significantly affect the drastic decrease by ovariectomy in uterine weight determined 30 days after operation, whereas the daily administration of 1 g/kg

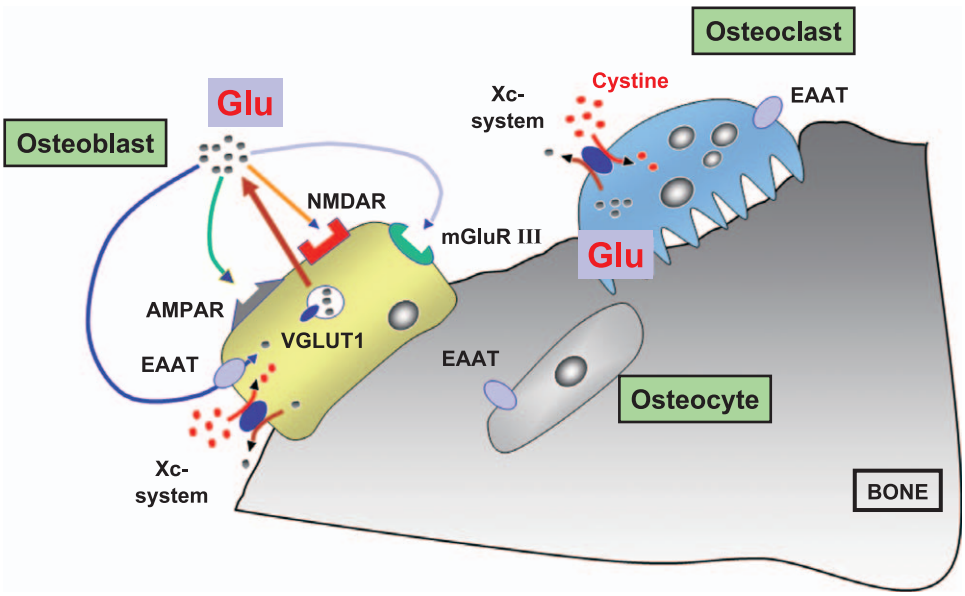

Fig. 2. Glutamatergic signaling machineries in bone. Glutamatergic signaling machineries are functionally expressed by the bone-forming osteoblasts, but absent from the bone-resorbing osteoclasts except for the EAAT isoforms. However, both cell types express the cystine/glutamate antiporter required for the biosynthesis of intracellular glutathione.

Glu for 28 days significantly increased Glu concentrations in tibial bone marrows irrespective of ovariectomy when determined 24 h after the last administration. Ovariectomy induced a significant reduction of bone mineral density in both total tibia and total femur when determined by single-energy X-ray absorptiometry at 30 days later. The daily administration of Glu for 28 consecutive days significantly prevented the reduction of bone mineral density in total tibia at a dose over 10 mg/kg and in total femur at a dose over 100 mg/kg, respectively. No significant alternation of bone mineral density was observed in total femur of sham-operated mice with the daily intraperitoneal administration of Glu at a dose of 1,000 mg/kg for 28 consecutive days (33).

Micro-CT analysis clearly showed marked bone loss in the cancellous bone, but not in the cortical bone, in ovariectomized mice at 30 days after operation, while the daily intraperitoneal administration of 1 g/kg for 28 days markedly prevented bone loss in the cancellous bone without affecting the cortical bone density on μCT analysis. In ovariectomized mice, moreover, a significant decrease was seen in bone volume/tissue volume (BV/TV) ratio with significant increases in the extent of eroded surface (ES/BS), the number of osteoclasts on bone surfaces (Oc no), and the extent of bone surface covered by osteoclasts (Oc surface) by histomorphometric analysis at 30 days after operation. However, ovariectomy did not significantly affect the extent of bone surface covered by osteoblasts (Ob surface). The daily administration of 1 g/kg Glu for 28 consecutive days was invariably effective in significantly preventing alterations of different bone parameters in ovariectomized mice when determined 30 days after operation.

Concluding remarks

The number of patients suffering from metabolic bone diseases is undoubtedly increasing in proportion to increasing life spans throughout the world, while drug therapy strategies are rather poor for the prophylaxis and treatment compared to those for other cardiovascular and neuropsychiatric disorders. One of the discouraging reasons is the absence of information about endogenous modulation mechanisms for both osteoblastogenesis and osteoclastogenesis. In disagreement with the prevailing view that glutamate modulates osteoclastogenesis through NMDA receptors expressed by osteoclasts (25 – 27), our present study has clearly demonstrated the complete absence of all glutamatergic machineries from osteoclasts differentiated from bone marrow precursor cells. In our hands, however, functional expression is seen in osteoblasts with glutamatergic signaling machineries, including NMDA (34), AMPA (35), and group III mGluR (36) receptor subtypes for the signal input; EAAT isoforms (37) for the signal termination; and VGLUT1 isoform (38) for the signal output (Fig. 2). Our findings about the new point of view on the prevention of bone loss by ovariectomy after the systemic administration of glutamate are therefore of a great interest and importance to wide spectra of basic and clinical scientists, not only in the bone biology field but also in the oxidative stress field. This review article highlights the promising prime development of "neuro-osteology" as an innovative and novel interdisciplinary field that will act as a scientific bridge between bone and brain biology. We believe that we should widely distribute information about innovative developments in this novel interdisciplinary field to other scientists in various disciplines of different scientific fields as quickly as possible. We have to dispatch our important experimental information to researchers involved in bone and brain biology who are performing investigations vital to assisting the elderly worldwise.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research to T.T. and Y.Y. from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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