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Review Article

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Glutamatergic regulation of bone resorption

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Abstract

There has been increasing evidence during the last years that glutamate (Glu), the major neuromediator of the nervous system, contributes to the local regulation of bone cell functions. Several classes of Glu receptors and transporters, as well as molecules involved in glutamate signal transduction in neuronal tissue, were identified in bone. While recent findings suggest that Glu may participate in mechanisms underlying bone formation, several studies indicate that Glu may also control bone resorption. Ionotropic NMDA and metabotropic Glu receptors are expressed by osteoclasts and electrophysiological studies have demonstrated that NMDA receptors (NMDAR) are functional on these cells. *In vitro* studies have shown that NMDAR are important for osteoclast function since several specific antagonists of NMDAR which block the current induced by Glu in these cells also inhibit bone resorption. Preliminary studies investigating the mechanisms of action of NMDAR antagonists on bone resorption are reviewed in this paper. There is also growing evidence that NMDAR are expressed throughout the osteoclastic differentiation sequence and that antagonists of NMDAR affect osteoclastogenesis. Very few *in vivo* studies have however investigated the role of Glu in skeletal metabolism and bone resorption and clearly further work is required to demonstrate the relevance of glutamate signaling in the physiology of bone resorption *in vivo*.

Keywords: Glutamate, NMDA Receptors, Osteoclasts, Bone

Introduction

Osteoclastic bone resorption involves the proliferation and differentiation of mononuclear preosteoclasts, their fusion to form multinucleated osteoclasts and the activation of mature osteoclasts to resorb bone. These different steps are controlled by systemic hormones and by a variety of local autocrine/paracrine factors, such as cytokines and growth factors produced in the microenvironment mainly by osteoblast lineage cells but also by immune cells, endothelial cells, and megakaryocytes[1,2]. In recent years, several reports have shown that bone is highly innervated and have identified receptors for neurotransmitters on bone cells, suggesting that neuro-osteogenic interactions may also be involved in skeletal metabolism[3,4]. This was well illustrated in the case of neuropeptides that are expressed in skeletal nerve fibers and have the capacity to regulate the activities of osteoclasts and osteoblasts[4]. The discovery on bone cells of receptors and transporters for glutamate, the most largely distributed neuromediator in the nervous system, has strongly supported a role for glutamate in the regulation of bone cell function. Molecules involved in glutamate signal transduction in neuronal tissue were also identified in bone, and there is now growing evidence that glutamate receptors are functional on osteoblasts and osteoclasts and are involved in both *in vitro* bone formation and resorption. This review will summarize the indications accumulated during the last three years for a role of glutamate on osteoclast differentiation and function.

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Glutamate Neurotransmission

Glutamate (Glu) is the principal excitatory neurotransmitter in the brain and as such is involved in a variety of physiological situations including development, memory, and learning[5,6]. Glu mediates these signals through activation of two types of membrane receptors, ionotropic Glu receptors (iGluR) which are gated ion channels and metabotropic Glu receptors (mGluR) coupled to protein G[7,8]. Three subtypes of iGluR have been classified according to their activation by specific agonists: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), Kainate (KA), and N-methyl-D-aspartate (NMDA). Overactivation of iGluR is also responsible for the neurotoxic actions of Glu, a

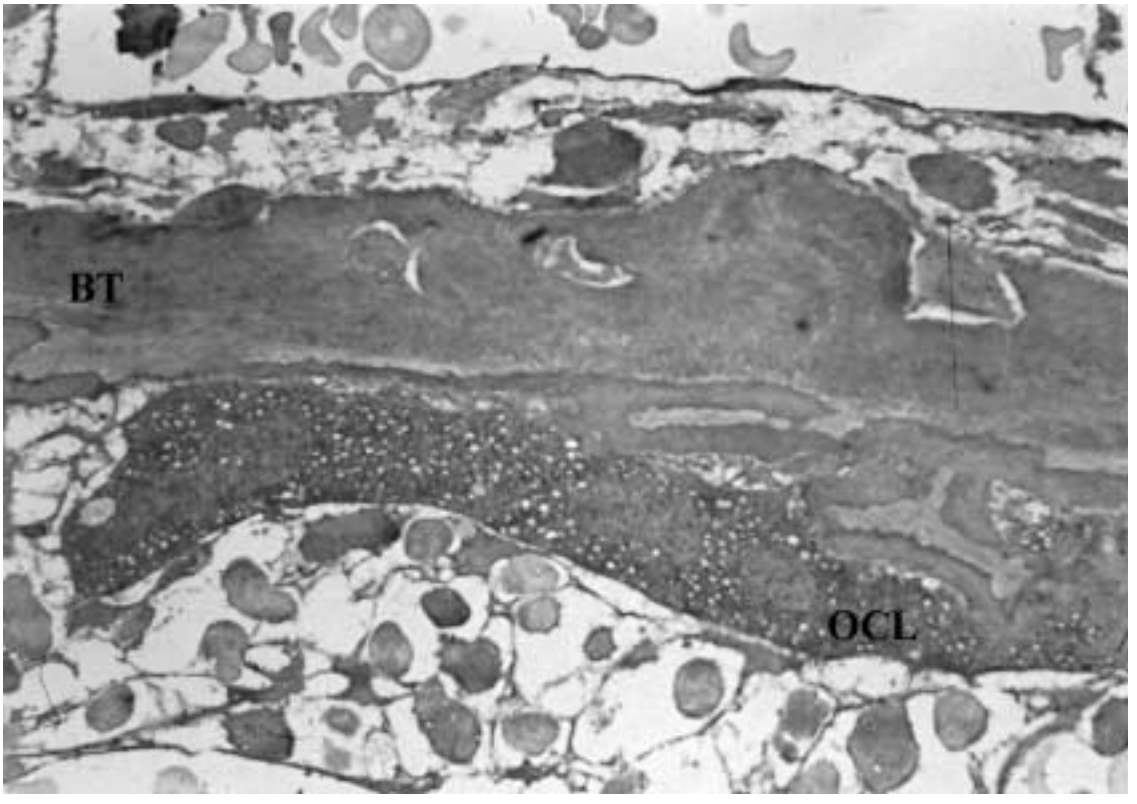

Figure 1. Immunostaining of rat bone section with NMDAR1 antibody. Osteoclast (OCL) in contact with bone trabeculae (BT) is highly stained. The staining is localized throughout the cytoplasm and excludes the nuclei (original magnification x 1000).

process termed excitotoxicity which is associated with the pathophysiology of hypoxic injury, stroke and epilepsy[9]. These physiological and pathological actions of Glu depend on the rapid reuptake of Glu from the synapse, which is achieved by glutamate transporter proteins present in the plasma membranes of both glial cells and neurons[10]. Three distinct, high affinity, sodium-dependent Glu transporters have been identified in the animal central nervous system (CNS), glutamate/aspartate transporter (GLAST), glutamate transporter 1 (GLT-1), and excitatory amino acid carrier 1 (EAAC1). At least five structurally distinct subtypes of human Glu transporters were described and characterized by molecular cloning, named excitatory amino acid transporters (EAAT1-5).

Synapses are highly organized to facilitate the transmission of signals from the presynaptic terminal to the postsynaptic membrane and to activate subsequent signal transduction cascades. This is the case for glutamate excitatory synapses where neurotransmission depends on the extremely precise distribution and organization of GluR at postsynaptic sites where they can respond to synaptically released Glu. Various studies have demonstrated that proteins containing PDZ domains, which interact with the cytoplasmic tails of GluR and connect them to the cytoskeleton, play an essential role in GluR postsynaptic clustering and function[11-13]. Synaptic transmission is terminated by Glu transporters, which rapidly lower the concentration of Glu in the synaptic cleft. Glu is transported primarily to glial cells where it is converted to glutamine by the glutamine synthetase pathway. Glutamine is released back to the neurons, where Glu is regenerated via glutaminase, a mitochondrial enzyme.

Glutamate signaling in bone cells

Over the last years, all the machinery required for Glu signaling in the CNS was identified in bone cells. The first description of Glu signaling in bone was reported by Mason et al.[14], who demonstrated the expression of GLAST mRNA in active cuboidal osteoblasts and osteocytes, that was regulated by mechanical loading of rat bone *in vivo*. GLT-1, another Glu transporter, was also present in bone marrow cells[14]. Two variants of GLAST were later identified in bone *in vivo*, but their roles in glutamate transport and signaling in bone cells have not been investigated to date[15].

The subsequent demonstration of the presence of all classes of GluR in bone cells has further supported a putative role for Glu in bone metabolism. The expression in bone of NMDA subtype of GluR (NMDAR) was the most studied. This hetero-oligomeric protein is composed of three classes of subunits, namely NMDAR1 (NR1), NR2 and NR3[8]. Highly active NMDAR channels are produced only

	RABBIT OSTEOCLASTS	MOUSE OSTEOCLASTS	RAT OSTEOCLASTS
NMDAR			
NR1	+	+	+
NR2A	-	+	*NI*
NR2B	+	+	*NI*
NR2C	-	-	*NI*
NR2D	+	+	*NI*
AMPAR			
GluR	+	*NI*	+
mGluR			
mGluR1	*NI*	*NI*	+
mGluR2/R3	*NI*	*NI*	+
PSD-95	+	+	*NI*

+=Present; -=Absent; NI=Non Investigated (see details in the text)

Table 1. Glutamate signaling in mammalian osteoclasts.

when the NR1 subunit is expressed together with one of the four NR2 subunits (NR2A to D). NR3 is a regulatory subunit that decreases the NMDAR channel activity[16]. Using immunocytochemistry on rat bone sections, we have shown that mature osteoblasts and osteoclasts as well as some osteocytes express NR1 subunit[17] (Figure 1). NR1 mRNA expression was subsequently demonstrated in bone and bone marrow cells using RT-PCR analysis and *in situ* hybridization[18]. Several other studies have confirmed that bone cells express NR1 together with various NR2 subunits, supporting a molecular diversity of these channels in bone similar to what was shown for brain[19-21]. The presence in bone of other iGluR, AMPA and Kainate receptors, was less studied but they were also identified in this tissue[17,22,23]. AMPA receptors (AMPAR) include GluR1-4 subunits while KA receptors (KAR) comprise GluR5-6-7, KA1 and KA2[24,25]. Similarly, expression of mGluR mRNAs was recently demonstrated in bone cells by RT-PCR analysis. Eight rat mGluR are divided into groups I to III on the basis of pharmacological properties and second messenger systems[26]. While mGluR1b (group I) and no other mGluR subtypes was identified in rat femoral osteoblasts[21], Hinoi et al.[20] have demonstrated the expression of group III mGluR (mGluR4 and mGluR8) in primary osteoblasts isolated from rat calvaria. A recent preliminary study using immunocytochemistry on rat bone sections showed that cells of the osteoblast phenotype express mGluR5 (group I) and mGluR8 while mGluR1 and mGluR2/3 (group II) are expressed by osteoclasts[27].

Other components of Glu signaling were described in bone. Cells expressing glutamine synthetase were observed in bone marrow using immunocytochemistry (our unpublished results). The NMDAR clustering protein PSD-95 mRNA was demonstrated in bone marrow cells, osteoblastic cell lines and mammalian osteoclasts[18,19]. Finally, Glu itself was identified in vesicles in osteoblasts from where it can be released by exocytosis[28], and was detected in the network of nerve fibers running in bone marrow in the vicinity of bone cells[3].

From all these studies, it is clear that several classes of receptors and transduction proteins required for glutamate signaling in neuronal cells are present in osteoclasts, suggesting a role for this neuromediator in osteoclast function. Table 1 draws up the components of Glu signaling specifically expressed by mammalian osteoclasts.

Osteoclasts express functional NMDA receptors

The high expression of NR1 subunits on osteoclasts lining on bone surfaces (Figure 1) suggested that NMDAR are functional in these cells. Different experimental approaches were used to investigate whether osteoclasts express active NMDAR. Since functional NMDAR require the presence of both NR1 and NR2 subunits, the molecular composition of NMDAR was analyzed in osteoclasts. Using RT-PCR analysis and *in situ* hybridization, we have demonstrated that mammalian osteoclasts express NR1 as well as NR2B and NR2D subunit mRNA[19], suggesting that active NMDAR channels in these cells may be produced by the association of NR1 with NR2B or NR2D. The presence of NR2A and NR2C subunit mRNA in osteoclasts has been the subject of controversy. The expression of these subunits may be more dependent on the age of osteoclasts and the species from which they originate. NR2A mRNA is preferentially expressed by mouse osteoclasts (personal observation). The

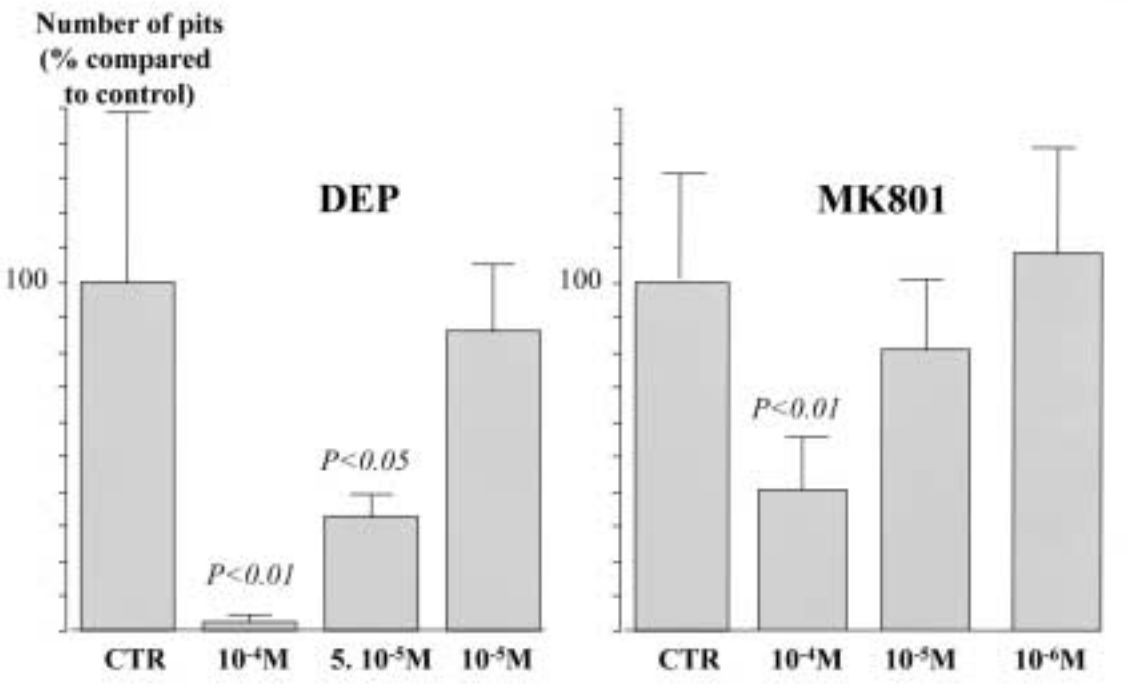

Figure 2. Effects of NMDAR antagonists on *in vitro* bone resorption. Two antagonists of NMDAR (DEP and MK801) were added at different concentrations (10^{-4}M to 10^{-6}M) to rabbit osteoclasts cultured during 24 hours onto cortical bone slices. The number of resorption pits per slice was scored and results are expressed as mean ± SD (percentage of control) of three experiments performed in quadruplicate.

expression of NR1 and NR2D proteins by rabbit osteoclasts was confirmed by immunofluorescence studies[19], while the presence of NR2A,B,C proteins was not investigated due to a lack of good specific antibodies directed against those subunits.

The presence in osteoclasts of specific binding sites for NMDAR ligands was also demonstrated. Binding experiments using radioactive MK801 [(5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine], an NMDAR antagonist that acts on the receptor channel site when opened, have shown that this antagonist specifically binds to osteoclasts[29]. The best proof that NMDAR are functional in osteoclasts was however brought by electrophysiology studies. Using the patch clamp technique, two agonists of the NMDAR, Glu and NMDA, were shown to activate whole cell currents recorded in isolated rabbit osteoclasts[30]. Functional NMDAR were observed in approximately 30% of mature osteoclasts. The currents induced by Glu and NMDA had electrophysiological and pharmacological properties similar to those documented for neuronal cells. Currents were sensitive to specific blockers of NMDAR, such as magnesium ions, MK801 and DEP [1-(1,2-diphenylethyl)piperidine]. The blocking of Glu and NMDA-induced currents by these specific antagonists was voltage-dependent, as expected for this type of current. The demonstration of Glu-induced currents in mature rabbit osteoclasts was later confirmed by Peet et al.[31]. It has been previously shown in neuronal cells that NMDAR containing NR1/NR2D constitute receptors with unique properties, including very slow deactivation kinetics of Glu and NMDA-induced currents. The features of NMDAR-mediated currents elicited in osteoclasts by application of Glu displayed this property, indicating that a major NR1/NR2D composition seems to be present in these cells[19]. However, in contrast to NR1 which is expressed ubiquitously in the brain, NR2 subunits show a distinct spatial and temporal expression pattern. As shown in synapses, it is therefore possible that a selective change in osteoclast surface expression of distinct NR2 subunits occurs depending on cell activation state. The relationship between expression of particular functional NMDAR by osteoclasts and their activity of bone resorption will have to be determined.

Effects of glutamate on *in vitro* bone resorption

Glutamate is a non essential amino acid used for cell metabolism, and it is consequently present in cell culture media and serum. It is therefore difficult to control its level in cell culture experiments and to study the effects of Glu and other agonists on *in vitro* bone cell function. This is the reason why most of the studies performed *in vitro* to investigate the role of glutamate on osteoclast formation and activity used antagonists of glutamate receptors, and particularly noncompetitive ones. We were the first to show that NMDAR are important for osteoclast activity using a monoclonal antibody directed against the NR1 subunit, as well as

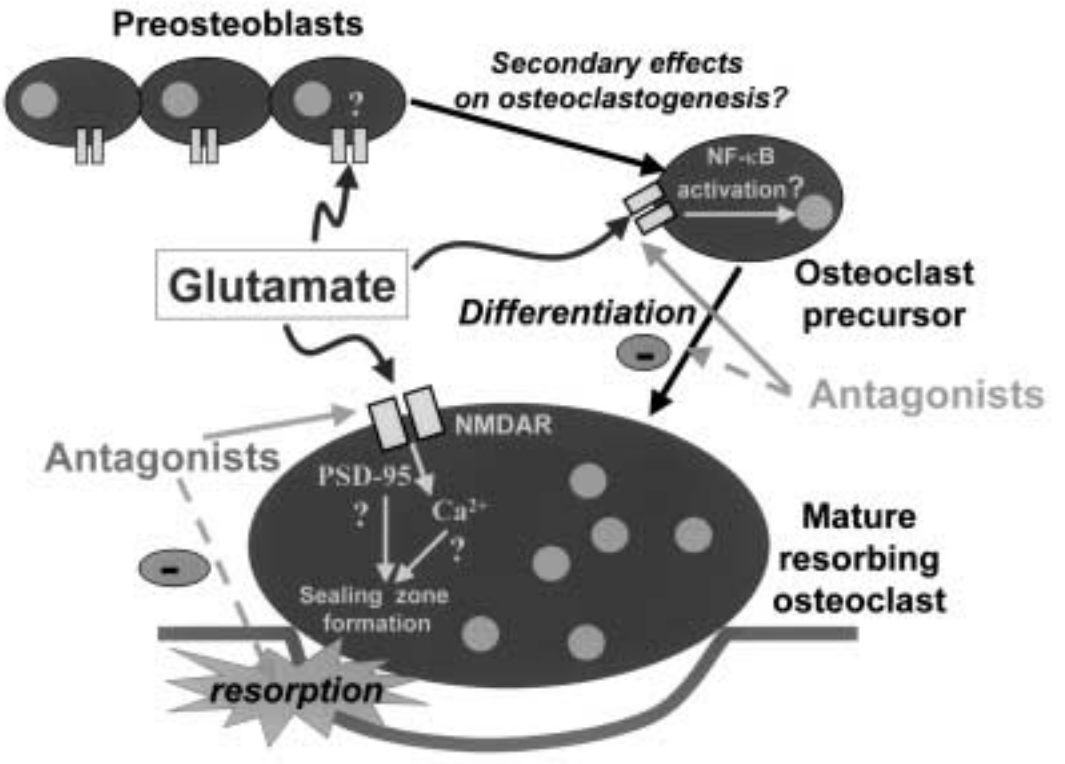

Figure 3. Possible mechanisms of action of glutamate on osteoclast formation and activity. Glutamate regulates osteoclast formation and function by acting on NMDAR present on osteoclast precursors and mature osteoclasts. By acting on NMDAR expressed by osteoclast precursors, glutamate may induce the NF-kB signaling pathway necessary for osteoclastogenesis. NMDAR present in mature osteoclasts appear to be involved in the process of adhesion-induced formation of the sealing zone required for bone resorption, possibly through the induction of changes in internal calcium concentrations or through their interactions with cytoskeleton proteins via PSD-95. The possibility that glutamate also controls osteoclastogenesis through an action on NMDAR expressed by preosteoblasts cannot be ruled out. Antagonists of NMDAR inhibit both osteoclast differentiation and bone resorption.

specific antagonists of NMDAR, all of which inhibit *in vitro* bone resorption by rabbit osteoclasts[17,29] (Figure 2). Functional modulation of NMDAR can be achieved at different binding sites such as the glutamate recognition site which is a target for competitive agonists and antagonists, the phencyclidine site located within the ion channel, the co-agonist glycine binding site and a polyamine modulatory site[32]. All these sites represent targets for the development of specific uncompetitive and competitive NMDAR antagonists. We showed that several competitive and uncompetitive antagonists of NMDAR binding to the ligand, glycine and channel sites of the receptor inhibit bone resorption, but those directed against the channel site (MK801, DEP) were the most potent ones[29]. These antagonists dose-dependently reduced the number of resorption pits on cortical bone slices at concentrations which were similar to those used in neuroscience studies and, although quite high (50μM), had no effect on cell viability. Using a similar bone resorption assay, Gray et al.[33] confirmed that MK801 reduced the number of resorption pits on dentine, but it had no consistent effect on the area and volume of pits. D-AP-5, a competitive blocker of NMDAR, did not reduce the number of resorption pits in their studies. This was not surprising, as those experiments were performed in glutamate- and serum-containing media, conditions which are not suitable to investigate the effects of competitive antagonists[34,35]. In another study, Peet et al.[31] have shown no effect of MK801 on bone resorption using rat and rabbit mature osteoclasts or explants of mouse calvaria prelabeled with 45Ca, despite an effect of MK801 on membrane currents of mature osteoclasts. In this study, the authors have however demonstrated that MK801 reduced resorption of pits in cocultures of bone marrow cells and osteoblasts in which osteoclasts develop, by acting predominantly on osteoclast differentiation rather than on their activity. The reason for this discrepancy between studies regarding the stage of the osteoclastic differentiation sequence at which NMDAR antagonists are active is not yet very clear. NMDAR may be involved in both osteoclast formation and bone resorption. Our preliminary results[36] have confirmed that NMDAR are expressed throughout the osteoclastic differentiation sequence from osteoclast precursors to mature resorbing osteoclasts, and that antagonists of NMDAR affect both osteoclastogenesis and osteoclast activity.

The mechanisms of action of GluR antagonists on bone

resorption have been poorly investigated. By allowing a calcium influx into the cells, activation of NMDAR induces changes of internal calcium concentration and modifications of membrane potential, two events that were shown to relate to bone resorption[37]. Similarities between osteoclasts and neuronal cells were observed concerning intracellular signaling pathways induced by NMDAR activation. In neuronal cells, NMDAR can interact directly with proteins of the cytoskeleton, and their activation induces modifications of the cytoskeletal organization which are involved in many neuronal cell functions[38]. Indirect interactions between NMDAR and cytoskeleton proteins through specific intracellular PDZ-containing proteins such as PSD-95 have also been documented[11]. Osteoclast activity is dependent on their adhesion to bone surface and their subsequent cytoskeletal reorganization required for bone resorption. We have studied the effects of MK801 on these different steps leading to osteoclast activation[29], and showed that MK801 has no effect on osteoclast attachment to bone, while it rapidly decreases the percentage of osteoclasts with actin ring structures that characterize actively resorbing osteoclasts. As is the case for neuronal cells, NMDA channel activity may therefore be important for the cytoskeletal organization of the osteoclast and the sealing zone formation required for bone resorption. The presence in osteoclasts of PSD-95 suggests that NMDAR in those cells may be associated with cytoskeleton through this protein. Contradictory results were however obtained by Peet et al.[31], who did not show any effect of MK801 in disrupting actin rings in mature rabbit osteoclasts. The different experimental settings used in these studies may explain this inconsistency. NMDAR are regulated by the protein tyrosine kinase Src[39], and another similarity with the nervous system is that this kinase is highly expressed by osteoclasts and plays a critical role in their cytoskeleton organization[40]. Whether Src also regulates the activity of NMDAR in osteoclasts has however not yet been investigated. We did not observe any effect of MK801 on induction of osteoclast apoptosis at concentrations that inhibited bone resorption and in identical experimental conditions[29], while preliminary results by Mentaverri et al.[41] demonstrated an effect of MK801 on the induction of osteoclast apoptosis using cells isolated from older rabbits and experimental conditions for the evaluation of apoptosis that were very different from those used in the bone resorption assay.

While a direct effect of GluR modulators on mature osteoclasts is confirmed by the presence of functional NMDA receptors and by the induction of downstream signaling pathways following their activation, the question arises whether the effects of NMDAR on osteoclastogenesis are direct or indirect. Osteoclast progenitor proliferation, differentiation and fusion to form terminally differentiated osteoclasts are controlled by stromal cells and osteoblastic cells that also express NMDA receptors[17,18]. No study has yet been performed to discriminate between direct effects of GluR modulators on osteoclast precursors and/or indirect effects through cells of the osteoblastic lineage. Arguments for direct effects on osteoclast progenitors were however suggested by our recent studies demonstrating the presence of NMDAR on osteoclast precursors isolated from mouse bone marrow as well as on RAW 264 cells, a murine myelomonocytic cell line that differentiates into osteoclasts under the influence of receptor activator of nuclear factor kB ligand (RANKL)[36]. We showed that antagonists of NMDAR inhibit the differentiation of RAW 264 cells into osteoclasts in the presence of RANKL, and our preliminary results indicate that Glu activates the NF-kB signaling pathway required for osteoclastogenesis in non differentiated RAW 264 cells (Merle et al., in preparation). Previous studies by Malone et al.[42] had already shown a long time ago that human peripheral monocytes express receptors for neuroexcitatory amino acids such as glutamate, that may be involved in their chemotaxis responses to the neuromediators. On the other hand, GluR are also functional in osteoblasts[21,43], and we cannot exclude that GluR antagonists also act on osteoblastic cells to modulate *in vitro* osteoclast formation in co-culture models containing osteoclast precursors and osteoblastic cells. Our hypotheses related to the mechanisms of action of glutamate in bone resorption are illustrated in Figure 3.

In vivo studies

Very few *in vivo* studies have investigated the role of glutamate in skeletal metabolism and bone resorption. Glu may act in part in an autocrine/paracrine manner, but the possibility for Glu to play a role as a neuromediator in bone has also been indicated. The importance of Glu as a signalling molecule between bone cells was demonstrated by the regulation of Glu transporters and receptors after mechanical loading of rat bone. GLAST expression was markedly increased in periosteal osteoblasts lining on surfaces where loading had induced bone formation, while absent on quiescent periosteal surfaces[14]. Gilbert et al.[27] reported a loading-induced loss of immunoexpression for mGluR5 in cells of the osteoblastic phenotype, while expression of other mGluR was not affected by mechanical loading. However, no study has yet shown any regulation of GluR expression on osteoclasts by mechanical loading of bone. Systemic administration of glutamate agonists and antagonists is difficult due to the impossibility to specifically target bone. Compounds that do not cross the blood-brain barrier were recently developed and studies investigating their effects on bone formation and resorption are conceivable. Preliminary results by Taylor et al.[23] have demonstrated that injection of Wistar rats with AMPA and Ka GluR antagonists before a period of mechanical loading, and then daily for 2 weeks, induced modifications of the amount of bone formed. These studies indicate that it may be possible to modulate the response of bone to mechanical loading *in vivo*, using specific antagonists of different GluR subtypes.

The hypothesis that Glu may act as a neuromediator in bone was proposed following the immunodetection of Glu in nerve fibers running in bone in the vicinity of bone cells[3].

Although Glu and its receptors may be involved in nociceptive transmission, as already shown in other peripheral tissues, the demonstration of contacts between Glu-immunoreactive nerve terminals and bone cells suggests that Glu released by skeletal nerve fibers may contribute to the regulation of bone cell activity. Localization of axons containing the neuropeptides substance P and calcitonin gene-related peptide in contact with osteoclasts near the trabecular bone was previously demonstrated[44,45]. Our group has recently shown that sciatic neurectomy and ovariectomy in rats induce a bone loss in tibiae associated with a reduction of Glu-containing nerve profile density, suggesting that glutamatergic nerve fibers may play a role in bone loss during immobilization and in osteoporosis[46,47]. Sciatic neurectomized and ovariectomized rats are two osteopenia models characterized by different mechanisms of bone loss. Decrease in bone formation rather than increase in bone resorption is characteristic after sciatic neurectomy, while estrogen deficiency in the model of ovariectomized rats results in an increase in bone turnover[48,49]. Although no histomorphometric evaluation of bone cell activity was performed in these animals, the coincidence of a decrease in glutamatergic nerve profile density and osteopenia suggests that innervation would preferentially exert a positive control on bone formation. Clearly, further studies are necessary to sustain this hypothesis and to attribute a role for glutamate in the bone loss induced in these models.

Many knockout of GluR subunits and Glu transporters have already been performed, resulting in neonatal death. For most of them the bone phenotypes were not analyzed, except in mice with NR1 and GLAST Glu transporter knocked out. The NR1 knockout mice did not show apparent abnormal skeletal phenotype[31,50], and in mice lacking GLAST Glu transporter no differences in mandible and long bone size, morphology, trabeculation, and bone areas of resorption and formation were detected compared with wild-type siblings[33]. However, since bone cells express different glutamate receptors and transporters, several pathways for glutamatergic signaling exist in this tissue that may compensate for each other. It is also possible that the essential roles of glutamate signaling components in bone may be revealed in functional studies, when skeletal homeostasis is challenged either by ovariectomy or by denervation. Selective deletion of glutamate receptors and transporters in bone will also be a very useful approach to investigate the role of these components in this tissue.

Clearly *in vivo* experiments addressing the importance of glutamate in bone metabolism are still lacking and it is therefore presently difficult to reconcile the *in vitro* effects of glutamate on bone resorption with those preliminary studies *in vivo*.

Conclusions

It is clear from all the results presented in this review that osteoclasts express several components of glutamate signaling that are linked to the regulation of osteoclast formation and bone resorption. Glutamate is a neuromediator that is widely expressed in the nervous system, but also in non-neuronal tissues where it can affect the function of a variety of cells, such as megakaryocytes in the bone marrow, keratinocytes in skin, and β-islet cells in the pancreas[51]. Bone is presently a major non-neuronal tissue where glutamate seems to play an important role. Although the origin of glutamate in this tissue is still debated, the presence of glutamatergic nerve fibers in bone[3] and the effects of glutamate on bone cell functions both contribute to the hypothesis of a neural control of skeletal turnover. Nerve fibers were identified in close contact to osteoclasts[3,44,45], and experimental denervation studies were shown to impair bone resorption[52,53], suggesting that osteoclast formation and function may be regulated by skeletal nerve fibers. An increasing number of studies has recently shown that bone contains many neuromediators that affect bone cell functions. Surprisingly, the same neuromediator is often able to modulate both osteoblast and osteoclast activities, as shown for example for vasoactive intestinal peptide[54]. This review illustrates that it is also the case for glutamate, which can regulate osteoclast function independently of its action on osteoblasts. These studies are however still preliminary and further work is required to determine the signaling cascade used by glutamate to regulate osteoclast formation and activity and to demonstrate the relevance of glutamate signaling in the physiology of bone resorption *in vivo*.

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