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ORIGINAL RESEARCH

Glutamate Receptor Agonists and Glutamate Transporter Antagonists Regulate Differentiation of Osteoblast Lineage Cells

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Abstract Development and function of osteoblast lineage cells are regulated by a complex microenvironment consisting of the bone extracellular matrix, cells, systemic hormones and cytokines, autocrine and paracrine factors, and mechanical load. Apart from receptors that transduce extracellular signals into the cell, molecular transporters play a crucial role in the cellular response to the microenvironment. Transporter molecules are responsible for cellular uptake of nutritional components, elimination of metabolites, ion transport, and cell–cell communication. In this report, the expression of molecular transporters in osteoblast lineage cells was investigated to assess their roles in cell development and activity. Low-density arrays, covering membrane and vesicular transport molecules, were used to assess gene expression in osteoblasts representing early and late differentiation states. Receptors and transporters for the amino acid glutamate were found to be differentially expressed during osteoblast development. Glutamate is a neurotransmitter in the central nervous system, and the mechanisms of its release, signal transduction, and cellular reabsorption in the synaptic cleft are well understood. Less clear, however, is the control of equivalent processes in peripheral tissues. In primary osteoblasts, inhibition of glutamate transporters with non-selective inhibitors leads to an increase in the concentration of extracellular glutamate. This change was accompanied by a decrease in osteoblast proliferation, stimulation of alkaline phosphatase, and the expression of transcripts encoding osteocalcin. Enzymatic removal of extracellular glutamate abolished these pro-differentiation effects, as did the inhibition of PKC- and Erk1/2-signaling pathways. These findings demonstrate that glutamate signaling promotes differentiation and activation of osteoblast lineage cells. Consequently, the glutamate system may represent a putative therapeutic target to induce an anabolic response in the skeletal system. Known antagonists of glutamate transporters will serve as lead compounds in developing new and specific bioactive molecules.

Keywords Osteoblast lineage cells · Anabolic response · Glutamate · Membrane transporter

Abbreviations

LDA	Low density array
GluT	Glutamate transporter
GluR	Glutamate receptor
SOS	L-serine-O-sulfate
THA	Threo-hydroxyaspartate
DHK	Dihydrokainic acid
L-CSA	L-cysteinesulfinic acid
L-HCSA	L-homocysteine sulfinic acid
S-DHPG	(S)-3,5-dihydroxyphenylglycine
TNFα	Tumor necrosis factor-α

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Introduction

Bone metabolism is largely controlled by osteoblast and osteoclast lineage cells [1]. Osteoblasts build the extracellular matrix of bone and control its mineralization, osteoclasts resorb the inorganic and organic phases of bone, and osteocytes, embedded in the bone matrix, act as mechanosensors [1, 2]. A wide range of chemical and physical signals derived from the extracellular matrix, neighboring cells, systemic and autocrine/paracrine factors, and mechanical load contribute to the regulation of development and activation of bone cells [3, 4]. Spatially restricted molecular action depends on the generation of confined structural compartments. These compartments hold locally secreted mediators in place, allowing for increase in extracellular levels that are physiologically relevant. In bone, these confined spaces to enable local effects are provided by the bone remodeling compartment (BRC) [1, 5, 6]. The BRC is characterized by a sheet of osteoblast-lining cells that seals off a small volume between bone surface and marrow space, shielding resorbing osteoclasts and bone-forming osteoblasts from the bone marrow [7]. Underneath this sheet of lining cells, a microenvironment for resorption and formation that meets the specific needs of the BRC is generated. Osteocytes extend their processes from the bone surface into canalicular spaces [8]. They form a dense network that allows for communication from the inside to the surface of the bone and the BRC, where the respective target cells, osteoblasts and osteoclasts, are located. Within the small volume of the canalicular system, the extracellular concentrations of mediator molecules were released by osteocytes, and subsequently, exerted autocrine and paracrine actions are well controlled [9].

Bone matrix and bone cell-derived factors are thought to be crucially involved in numerous physiological processes. Recently, osteocytes were found to be responsible for endocrine functions of bone, as they are the source of fibroblast growth factor 23 (FGF23), a "hormone" controlling homeostasis of phosphatemia and sclerostin, a factor that controls bone formation [10]. FGF23 and sclerostin, however, act systemically and on distant targets. Growth factors and cytokines that act locally within the BRC could be released during bone matrix resorption, which is the case for transforming growth factor beta (TGFβ) [11]. Alternatively, bioactive molecules can be released upon stimulation by specific triggers [11]. An example of locally restricted actions of soluble mediators is the release of neurotransmitters in the central nervous system (CNS) [12], which is controlled by a delicate interplay of transmitter release, ligand-induced signal transduction, and the essential removal of the transmitter by membrane transporters at the synaptic junction.

Despite the important roles of membrane transporters in homeostatic processes, their potential to serve as therapeutic targets has not been widely explored [13]. Biochemically, transporters are integral membrane proteins that allow the transfer of compounds, such as nutrients [amino acids (AAs), sugars, and vitamins], ions, and drugs across membranes. Based on their function as cellular gatekeepers, controlling the access of small molecules inside cells and organelles, membrane transporters are potential therapeutic targets for novel drugs [13]. In the current study, the expression of membrane transporter families was investigated in differentiating primary murine osteoblasts on low density arrays (LDAs). The LDAs were configured for the detection of transcripts encoding transporters of solute carrier (SLC) families, summarized in http://www.bioparadigms.org/; this website lists 52 SLC gene families with approximately 400 transporter genes, representing a major proportion of transporter-related genes. During the development of osteoblast lineage cells in vitro, glutamate transporters (GluT) were differentially expressed in cells, representing early and late differentiation stages. Previously, expression of GluT molecules was found to be increased in osteocytes embedded in the cortex of mechanically loaded bones [14, 15]. In the current study, the autocrine modulations of osteoblast proliferation and differentiation by glutamate are described, and the respective mechanisms of action have been partially elucidated.

Materials and Methods

Isolation and Culture of Primary Murine Osteoblasts

Primary murine osteoblasts were isolated from calvariae of one- and two-day-old *C57Bl/6J* mice (Charles River, Sulzfeld, D) by sequential collagenase digestion, as described previously [16]. Briefly, 25 calvariae were digested (5 × 20 min) in Hank's Balanced Salt Solution (HBSS; Sigma, Buchs, CH) containing 3 mg/ml collagenase II (Worthington, NJ, USA). Fractions 3–5 were pooled, and 10^6 cells were seeded into 75 cm^2 culture flasks and grown in culture medium containing α-minimum essential medium (αMEM) supplemented with 10 % fetal bovine serum (FBS; Inotech AG, Dottikon, CH), 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO BRL Life Technologies, Basle, CH) at 37 °C in a humidified atmosphere with 5 % CO_2. After 4 days in culture, cells were stored in liquid nitrogen (10^6 cells/ml). Before use, cells were thawed, grown in culture medium for 4 days, and used according to the experimental protocol; time periods

for preparation and culture of primary calvarial osteoblasts were strictly adhered to.

Assessment of Osteoblastic Gene Expression

To analyze gene expression, differentiated primary murine osteoblasts were seeded at a concentration of 20,000 cells/cm^2 into 24-well plates (2 cm^2/well) and cultured for 7, 14, and 21 days in medium supplemented with or without 50 μM ascorbic acid/10 mM β-glycerophosphate (βGP). Differentiation and mineralization of the cells were assessed by staining the cultures for alkaline phosphatase (ALP) and with Alizarin Red. Total RNA was isolated and prepared using the RNeasy Mini Kit (Qiagen, Hombrechtikon, CH), following the manufacturer's recommendations. Complementary DNA (cDNA) was produced from total RNA using AMV reverse transcriptase (Promega, Dübendorf, CH). The cDNA was placed on custom LDA cards (Applied Biosystems/Life Technologies, Dübendorf, CH) containing Assays on Demand (AoD; Applied Biosystems, Life Technologies, Dübendorf, CH) for ten housekeeping genes for standardization, 23 bone cell markers, and 156 molecular transporters (Suppl. Table 1). The amount of cDNA added was 3 ng/well; polymerase chain reaction (PCR) assays were performed on an ABI7900 System (Applied Biosystems/Life Technologies, Dübendorf, CH) with a thermal cycling profile of 2 min at 50 °C, 10 min at 94.5 °C, with 40 cycles of amplification (30 s at 97 °C and 1 min at 59.7 °C).

LDA data were subsequently confirmed by conventional PCR assays using AoD to quantify transcript levels for the GluT Slc1a1 (Mm00436590_m1), Slc1a3 (Mm00600697), Slc1a4 (Mm00444532_m1), and SLC1a6 (Mm011732 79_m1). For the detection of glutamate receptors (GluR), AoDs specific for mGluR1 (Mm01187084_m1), mGluR3 (Mm01316766_m1), mGluR4 (Mm01306128_m1), mGlu R5 (Mm00690332_m1), mGluR6 (Mm00841148_m1), mGluR7 (Mm01189424_m1), mGluR8 (Mm00433840_ m1), AMPA3 (Mm00497506_m1), Grina (Mm0045820 8_m1), Grina1a (Mm00724265_m1), and NMDA2D (Mm 00433822_m1) were used. For standardization, levels of transcripts encoding 18 s rRNA (Hs99999901_s1), glucuronidase-β (Gusb; Mm00446953_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Mm999999 15_g1) were determined. All PCRs were performed on an ABI7500 System (Applied Biosystems/Life Technologies, Dübendorf, CH) using a thermal cycling profile of 5 min at 95 °C with 40 cycles of amplification (30 s at 97 °C and 1 min at 59.7 °C).

Western Blotting

After specific treatment protocols as outlined in the Results sections, cells were collected and sonicated in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % NP-40, 1 % sodium deoxycholate, 1 % Triton X-100, 0.2 % SDS, 1 mM β-glycerophosphate, and 1 mM Na_3VO_4) supplemented with a protease inhibitor cocktail (Sigma, Buchs, CH). Cell lysates were centrifuged (15 min at 16,000×*g*), and the concentration of proteins was determined by Bradford assay (Bio-Rad, Cressier, CH). Samples were denatured in gel loading buffer (62.5 mM Tris/HCl pH 6.8, 10 % glycerol, 2 % SDS, 2.5 % β-mercaptoethanol, 0.01 % bromophenol blue) for 5 min at 95 °C. Equal amounts of protein (50 μg) were loaded onto each lane and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (150 mV, 2 h) on pre-cast 8–16 % gradient gels (Bio-Rad, Cressier, CH). Proteins were then transferred onto nitrocellulose membranes (Bio-Rad, Cressier, CH) by using a half-dry electroblotting machine (Bio-Rad, Cressier, CH). Nonspecific binding sites were blocked with 5 % (w/v) nonfat milk powder in 20 mM Tris/150 mM NaCl/0.1 % Tween 20. The membranes were probed overnight in 4 °C by incubation with the primary rabbit antibodies directed against Slc1a1, Slc1a3, and GluR2/3/4 and antibodies against phosphorylated Erk1/2 (p-Erk1/2), Erk1/2, phosphorylated PKC [p-PKC(pan)] (Cell Signaling, Allschwil, CH), and PKC(pan) (Acris Antibodies GmbH, Herford, Germany), respectively. Subsequently, membranes were incubated with secondary goat anti-rabbit antibodies labeled with IRDye800CW and IRDye680LT (Li-Cor Bioscience, Lincoln, NE, USA). Visualization of labels was achieved using an Odyssey® CLX Infrared Imaging System (Li-Cor Bioscience, Lincoln, NE, USA).

Inhibition of Glutamate Transport

To assess the function of GluT in osteoblast lineage cells, inhibitors of GluT (Sigma-Aldrich, Buchs, CH) were added to cultures of primary murine osteoblasts. Cells were seeded into 96-well plates at a density of 2000 cells/100 μl and grown for 3–5 days in NEUROBASAL glutamate-free medium (Invitrogen/Life Technologies, Dübendorf, CH) supplemented with L-serine-O-sulfate (SOS; 200, 400, and 800 μM), DL-threo-hydroxyaspartate (THA; 500, 1000, and 2000 μM), and (2S,3S,4R)-2-carboxy-4-isopropyl-3-pyrrolidineacetic acid (DHK; 200, 400, and 800 μM). Glutamate transport was blocked with nonselective and selective inhibitors: nonselective inhibitor SOS; nonselective inhibitor THA; and DHK, a selective inhibitor of EAAT2 (Slc1a2) [17, 18].

Determination of Viable Cells and of ALP Activity

To determine cell numbers and the ALP activity, cells were seeded into 96-well plates (2000 cells/100 µl cell culture medium) and cultured for 3–5 days. Viable cells were determined using a Cell Proliferation Kit II (Roche Diagnostics, Basle, CH) following the manufacturer's instructions. To assess the ALP activity, cells were lysed with 25 µl 0.1 % Triton X-100 and frozen at −20 °C. Subsequently, 50 µl of ALP substrate [1 M diethanolamine pH 9.8, 1 mg/ml p-nitrophenyl phosphate (pNPP)] was added to the lysates. After 30 min, reactions were stopped through the addition of 50 µl 0.1 M EDTA (pH 8.0). Absorption was measured at 470 nm to assess viable cells and at 405 nm for ALP (reference wavelength 690 nm), using an Infinite200Pro spectrophotometer (Tecan Group Ltd., Männedorf, CH). The specific culture conditions are described in the "Results" section.

Measurement of Extracellular Glutamate Concentration

To assess the effects of GluT inhibitors on the concentrations of extracellular glutamate, supernatants of osteoblasts either treated or nontreated with inhibitors of GluT were collected, and glutamate content was determined using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit (Invitrogen, LuBio Science GmbH, Lucerne, CH). In brief, cells were grown in NEUROBASAL glutamate-free culture medium for up to 72 h in the presence of GluT inhibitors, and the medium was collected after 36, 54, and 72 h. The concentration of glutamate in the collected samples was determined following the manufacturer's instructions.

Regulations of Gene Expression by SOS and THA

Primary murine osteoblasts, seeded as described above, were treated with 400 µM SOS and 1000 µM THA or solvent, and cultured in NEUROBASAL glutamate-free culture medium for 5 days. Levels of transcripts encoding osteocalcin (Mm00432271_m1), collagen I (Mm00801666_g1), and ALP (Mm00475831_m1) were quantified. The analysis was performed on an ABI7500 System with a thermal cycling profile of 5 min at 95 °C with 40 cycles of amplification (30 s at 97 °C and 1 min at 59.7 °C).

Effects of GluR Agonists on Osteoblast Lineage Cells

To investigate the effects of GluR agonists on primary osteoblasts, extracellular glutamate was removed by enzymatic degradation with glutamate pyruvate transaminase (GPT; Roche Diagnostics, Basle, CH) [19–21]. For this purpose, media were treated before use for 1 h at 37 °C with GPT (5 U/ml)/100 µM pyridoxal-L-phosphate/10 mM pyruvate to remove glutamate. Subsequently, primary murine osteoblasts were seeded at a density of 10^4 cells/cm^2 and the culture medium was replaced every 24 h for up to 5 days. Culture media were supplemented with SOS (200, 400, 800 µM) or either one of the GluR agonists L-CSA (25, 100, 400 µM), L-HCSA (12.5, 50, 200 µM), and S-DHPG (25, 100, 400 µM), all purchased from Sigma-Aldrich, Buchs, CH. The number of viable cells and ALP activity were determined after 3 and 5 days of culture.

Elucidation of Signaling Pathways Activated by Glutamate

To characterize the signaling pathways activated by glutamate, specific inhibitors of Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), and the mitogen-activated protein kinases Erk1/2 were added to cultures of primary murine osteoblasts grown in medium supplemented with SOS (400 µM). For inhibitors of CaMKII—PI3K, PKC, and Erk1/2—SCP0001 (0.1, 0.2, and 0.4 µM); W1628 (0.1, 0.2, and 0.4 µM); P3115 (4, 8, and 16 µM); and U01264 (0.1, 0.2, and 0.4 µM) were used, respectively (Sigma-Aldrich, Buchs, CH). After 3–5 days, the number of viable cells and ALP activity were determined.

Statistical Analysis

Values are expressed as the mean ± SD. Statistical comparison was evaluated by one-way ANOVA with Bonferroni's post hoc test using GraphPad Prism version 6 for Windows, The level of significance was established at $p < 0.05$ and $p < 0.01$, respectively.

Results

Expressions of GluT and GluR in Osteoblast Lineage Cells

Primary mouse osteoblast lineage cells were isolated from calvariae of one- to two-day-old *C57/B6J* mice and grown with vitamin C, with or without βGP. Cell differentiation was assessed by ALP and Alizarin Red staining (Suppl. Fig. 1). Expression of ALP was detected within 1 week, and levels increased during the further culture period, while Alizarin Red staining was detected after 3 weeks. Assessment of gene expression using LDA revealed that transcripts encoding GluR, GluT, and vesicular GluTs (VGluT) were expressed during osteoblast differentiation, which

was subsequently confirmed by western blotting analysis (Fig. 1). Expressions of GluTs, Slc1a1 and Slc1a3, and the GluR GluR2/3/4 were demonstrated in primary osteoblast lineage cells and in the osteoblast cell line MC3T3-E1.

Expression of Transcripts Encoding GluT and GluR Osteoblast Lineage Cells

Primary osteoblasts from *C57Bl/6J* mice support the development of osteoclasts in co-cultures with hematopoi etic precursors when incubated with 10^{-8} M $1,25(OH)_2D$ and 0.5 ng/ml TNFα. Under these conditions, the expression of mRNA encoding GluT and GluR family members was investigated. Levels of transcripts encoding the transporters, Slc1a1 and Slc1a3, were increased by $1,25(OH)_2$VitD and reduced by TNFα. Levels of transcripts encoding Slc1a4 and SLC1a6 were affected to a lesser extent (Fig. 2). Transcripts encoding the GluRs AMPA3, Grina1a, and NMDA2D were downregulated by TNFα and the level of mRNA encoding the GluR Grina was increased following treatment with $1,25(OH)_2D$ (Fig. 3). Levels of transcripts encoding GluR family members mGluR1-8 were not modulated by $1,25(OH)_2D$ and TNFα (data not shown).

Effects of Inhibitors of GluT on the Differentiation of Osteoblasts

After demonstrating the expression of GluT on osteoblast lineage cells, the effects of glutamate and the role of GluTs were investigated. Cells were cultured for 5 days in media supplemented with inhibitors of GluT; thereafter viable cells and the ALP activity were determined. Treatment of cultures with SOS (200–800 μM) led to a decrease in the number of viable cells and an increase in specific ALP activity (Fig. 4a), similar to the effects observed with $1,25(OH)_2$VitD (data not shown). Treatment with THA (500–2000 μM) caused a decrease in the number of viable cells and an increase in the ALP activity, similar to the effects observed with SOS, while DHK did not affect the cell viability or the ALP activity (data not shown). For the further characterization of the roles of GluT on osteoblast development and activity, the modulation of the levels of transcripts encoding osteocalcin (Fig. 4b), ALP (Fig. 4c), and collagen I (Col I; Fig. 4d) by GluT inhibitors was assessed. In cells exposed to SOS (400 μM) and THA (1000 μM), levels of mRNA encoding osteocalcin and ALP were significantly increased, while Col I mRNA was downregulated. DHK caused a small but significant increase in ALP transcript levels, but did not affect osteocalcin and Col I mRNA.

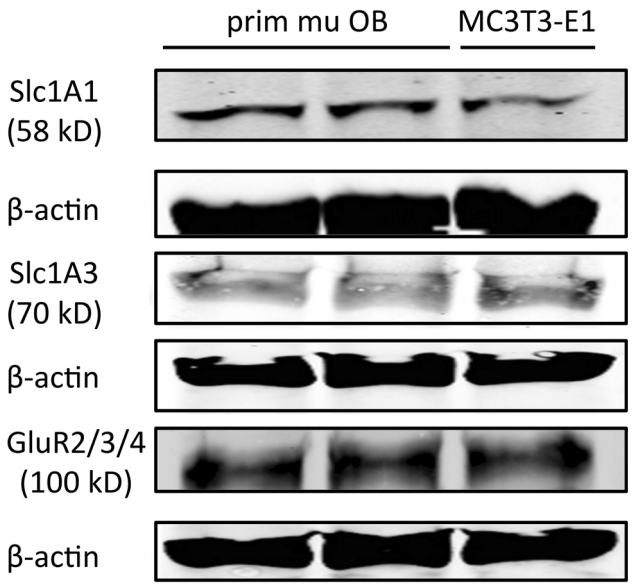

Fig. 1 Detections of GluR and GluT by western blotting. Mouse primary osteoblasts and the osteoblastic cell line MC3T3-E1 express Slc1a1, Slc1a3, and GluR2/3/4

Inhibition of GluT Leads to an Increase in Extracellular Glutamate

To assess the capacity of GluT to control the concentration of extracellular glutamate, primary osteoblasts were treated with the GluT antagonists: SOS, THA, and DHK. Exposure of osteoblasts to SOS (200, 400, 800 μM; Fig. 5a) and THA (0.5, 1, 2 mM; Fig. 5b) led to dose- and time-dependent accumulation of extracellular glutamate over 72 h, while treatment of the cells with DHK (200, 400, 800 μM; Fig. 5c) did not affect levels of extracellular glutamate.

Glutamate and GluR Agonists Stimulated Osteoblast Differentiation

Inhibition of GluT increased extracellular glutamate and induced osteoblast differentiation. Therefore, the direct effects of glutamate on osteoblast lineage cells were determined. Addition of glutamate (250, 500, 1000 μM) to osteoblast cultures caused a dose-dependent decrease in the number of viable cells (Fig. 6b) and an increase in ALP activity (Fig. 6a) in a dose-dependent manner.

Due to its structural similarity with glutamate, SOS acts both as a GluT antagonist and as a GluR agonist. To elucidate whether GluR agonists mimic the effects of glutamate, osteoblasts were grown in the presence of GPT to degrade cell-derived glutamate (Suppl. Fig. 2) in media supplemented with SOS (200, 400, 800 μM), and with the exclusive GluR agonists: L-CSA (25, 100, 400 μM), L-HCSA (12.5, 50, 200 μM), and S-DHPG (25, 100, 400 μM). Treatments with SOS and L-CSA resulted in increased extracellular glutamate, the levels of which were

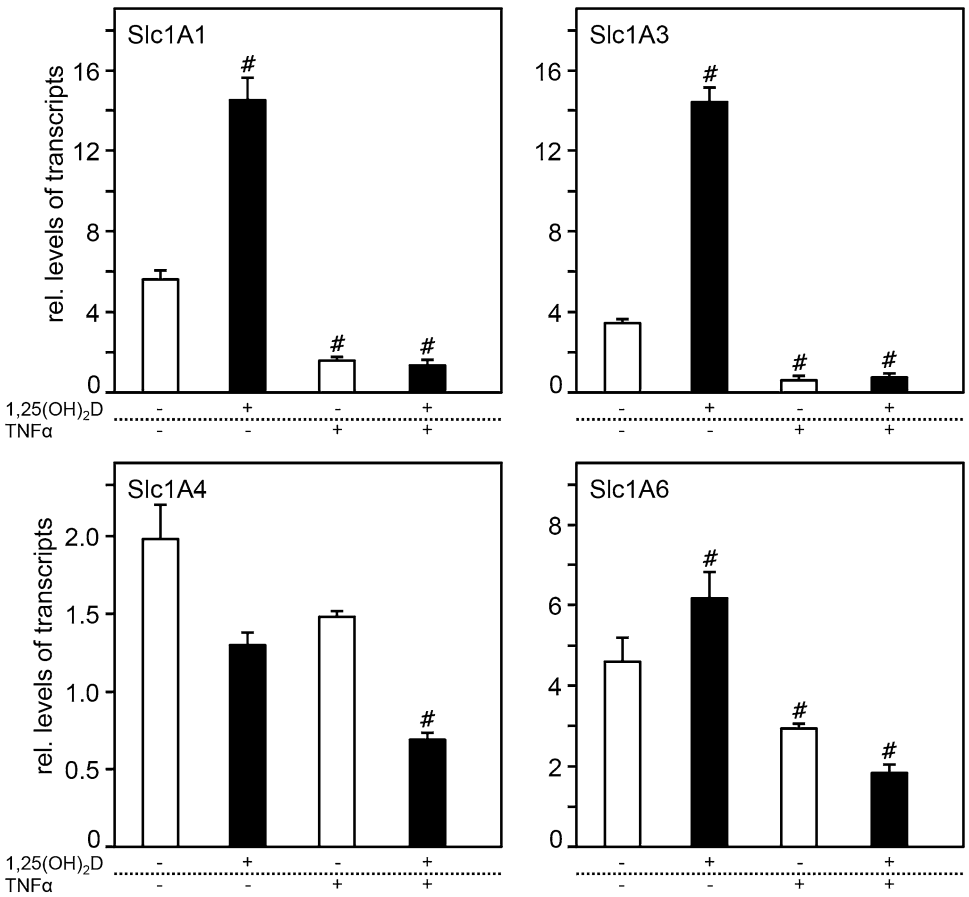

Fig. 2 Analysis of levels of transcripts encoding GluT by real-time PCR. Levels of transcripts encoding GluT were regulated by $1,25(OH)_2D$ and TNFα in primary osteoblast lineage cells. Levels of mRNA for Slc1a1 and Slc1a3 were increased by $1,25(OH)_2D$ alone, whereas treatment with TNFα led to a decrease in mRNA levels. Levels of transcripts encoding Slc1A4 and Slc1A6 were decreased mainly when $1,25(OH)_2D$ and TNFα were added to the cultures. Values are expressed as mean ± SE ($n = 3$; $^{\#}p < 0.05$ vs. control)

attenuated by GPT. SOS (Fig. 6c, d) and L-CSA (not shown) were found to stimulate the differentiation of osteoblast lineage cells as well as in the presence of GPT. L-HCSA exerted similar effects, while no such effects were observed with S-DHPG (data not shown).

Glutamate Signals Through the PKC and Erk1/2 Pathways

After demonstrating the effects of glutamate and SOS on the development of primary murine osteoblasts, the respective signaling cascades were analyzed. The inhibitors SCP0001 (CaMKII), W1628 (PI3K), P3115 (PKC), and U0126 (Erk1/2) were used to block specific signal transduction pathways. Exposure of primary osteoblast lineage cells to SOS and P3115 or U0126 reversed the effects of SOS on cell proliferation and ALP expression. There was a dose-dependent increase in the number of viable cells and a reduction in the ALP activity (Fig. 7). Inhibitions of PI3K and CaMKII did not attenuate the cellular effects of SOS (data not shown).

Western blotting analysis confirmed the activation of PKC and Erk1/2 upon treatment of primary osteoblasts with SOS and glutamate within 30 min. Treatments of cells with 1 mM glutamate and 400 µM SOS led to the activations of PKC and ERK1/2, respectively (Fig. 8). Levels of p-PKC and p-ERK1/2 were increased after exposures to glutamate and SOS for 20 min (PKC) and 30 min (Erk1/2), respectively.

Discussion

In the current study, the expression and function of the glutamate signal molecular system (GSMS) were investigated in vitro in primary osteoblast lineage cells. Transcriptional expression analysis by LDA revealed that members of the GluT and GluR families were differentially expressed by osteoblast lineage cells during development. The functional relevance of these molecules was confirmed, as glutamate modulated osteoblast proliferation, and supported cellular differentiation.

Glutamate is the major neurotransmitter in the CNS. It is released into the synaptic cleft upon arrival of an action potential. Glutamate exerts its signal through different classes of GluR in the post-synaptic membrane, such as AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), NMDA (*N*-methyl-D-aspartate), kainate, and

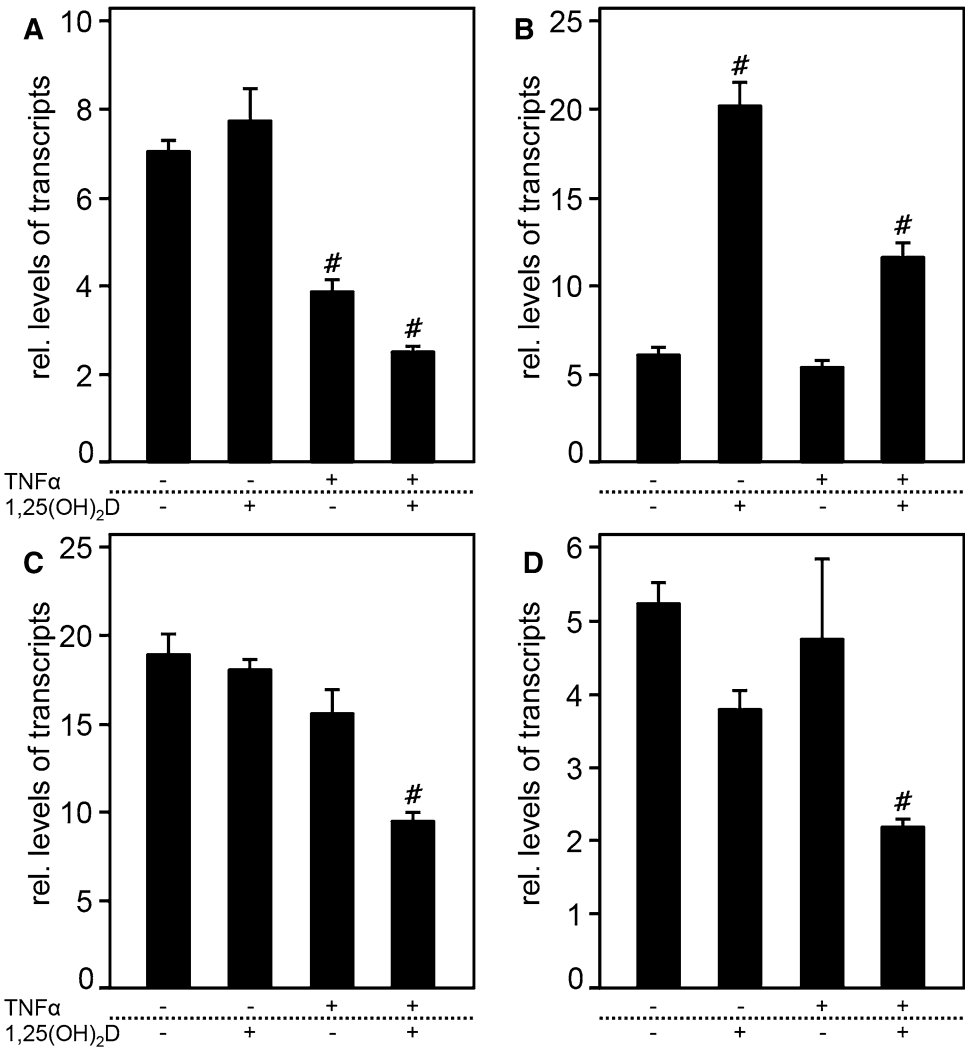

Fig. 3 Analysis of levels of transcripts encoding GluR by real-time PCR. Levels of transcripts encoding GluR were regulated by $1,25(OH)_2D$ and TNFα in primary osteoblast lineage cells. Levels of transcripts encoding AMPA3 (**a**) were decreased when the cells were cultured with TNFα, while those encoding Grina were increased in the presence of $1,25(OH)_2D$ (**b**). Levels of mRNA encoding Grinl1a (**c**) and NMDA2D (**d**) were significantly lowered only in cells grown with $1,25(OH)_2D$ and TNFα. Values are expressed as mean ± SE ($n = 3$; $^{\#}p < 0.05$ vs. control)

metabotropic (mGluR) receptors [22]. To terminate the synaptic action of glutamate, its concentration is reduced by diffusion and cellular uptake through GluT in the pre- and post synaptic membranes and in adjacent glial cells [23]. For controlled glutamate action, the following criteria need to be met: (i) release of glutamate into confined space to allow rapid and tightly controlled changes in concentration; (ii) availability to mediate a specific signal; and (iii) the presence of GluT to terminate glutamate signaling.

The expressions of GluT and GluR in osteoblast lineage cells corroborate previous findings and further support a physiological role for glutamate signaling in bone. It has been shown that osteocytes express GluT, and that these transporters are upregulated during mechanical load [14]. Furthermore, the BRC, osteocyte lacunae, and canaliculi provide the confined spaces required for the locally restricted action of small molecular weight osteoblastic products. Osteoblast lineage cells were known for a long time to release growth factors and cytokines that contribute to hematopoiesis or become part of specific niches at the bone–bone marrow interface [24]. Only in recent years, however, has the role of bone as an endocrine organ, controlling systemic and organ-specific functions, been fully appreciated [24]. To this effect, the role of osteocytes in the regulation of bone mass, through the release of the Wnt pathway inhibitor sclerostin, and osteocyte regulation of phosphatemia via the phosphate-regulating hormone FGF23, are new aspects of skeletal homeostasis [10]. The release of glutamate by osteoblast lineage cells and the expressions of GluT and GluR by these cells further open up new possibilities for autocrine and paracrine regulations of bone metabolism.

Experiments in the current study have revealed that glutamate, GluR agonists, and GluT antagonists exert differentiating effects on primary osteoblasts. These mechanisms allow glutamate to modulate the interplay between bone

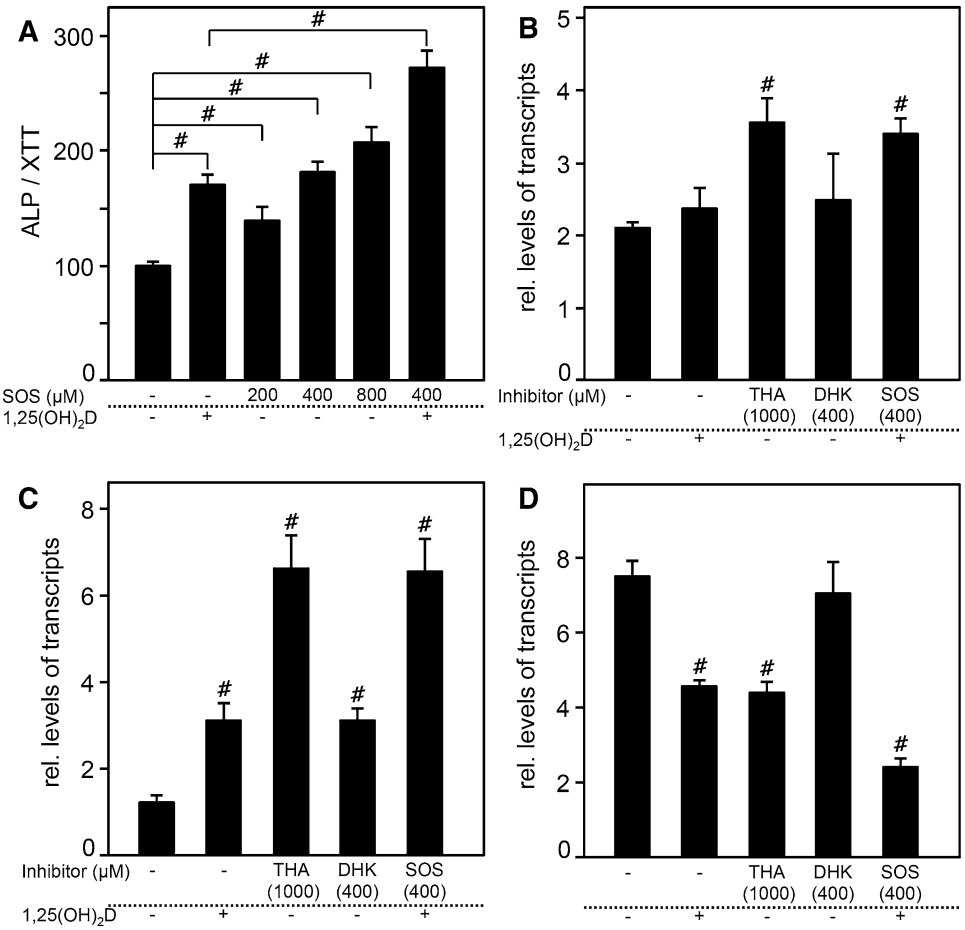

Fig. 4 GluT antagonists, SOS and THA, modulate differentiation and proliferation of osteoblast lineage cells. SOS led to a dose-dependent increase in the expression of ALP per cell number (**a**). Values are expressed as mean ± SE ($n = 6$; $^{\#}p < 0.05$ vs. control). Upon treatment with SOS and THA, levels of transcripts encoding osteocalcin (**b**), and ALP (**c**), were increased, while Col I mRNA levels **d** were decreased in osteoblast lineage cells. Treatment with DHK did not affect transcript levels. Values are expressed as mean ± SE ($n = 3$; $^{\#}p < 0.05$ vs. control)

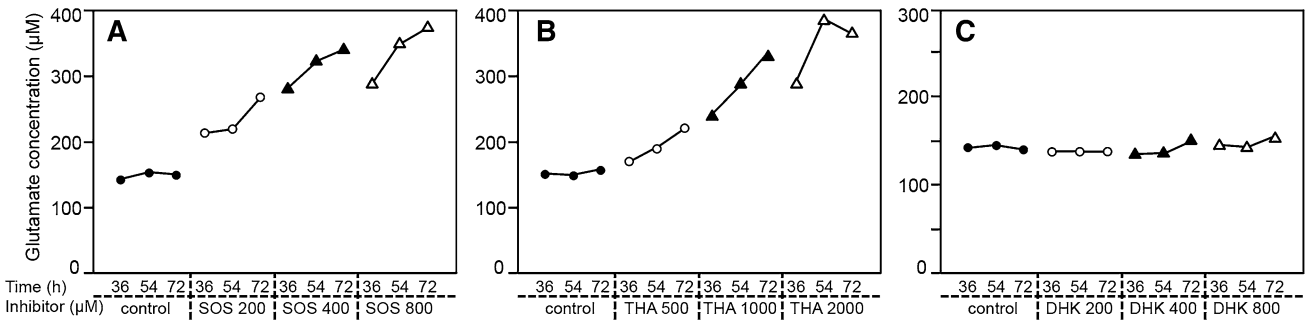

Fig. 5 Antagonists of GluT increase extracellular glutamate. Treatment of osteoblast lineage cells for 36, 54, and 72 h with SOS (**a**) and THA (**b**) dose-dependently increased extracellular glutamate levels. Treatment of the cells with DHK (**c**) did not lead to changes in glutamate concentration

resorption and formation. The physiological processes of bone modeling and remodeling are dependent on a functional communication network encompassing osteoblasts, osteoclasts, and osteocytes, which are controlled by local signals [25, 26]. Previous studies that have investigated the expression of GluT by osteocytes [14] and GluR expression by osteoblasts [27, 28], however, were limited as they focused on specific members of GluT and GluR families and did not aim to describe a complete expression profile of the GSMS in osteoblast lineage cells. In the current study, mouse primary osteoblasts were found to express at least five GluTs (Slc1a1-a5), four ionotropic GluRs (AMPA, NMDA), and seven metabotropic GluRs (GluR1-7). Glutamate and various GluR agonists and GluT antagonists were found to

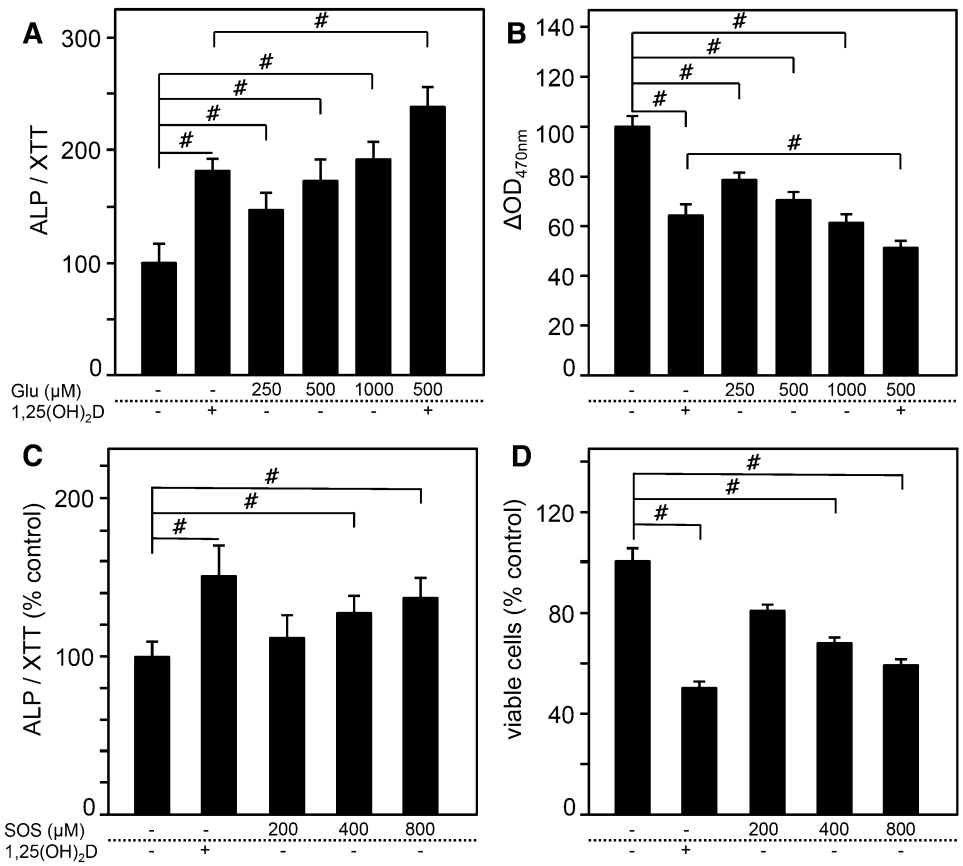

Fig. 6 The role of glutamate signaling in osteoblast differentiation and proliferation. Exposure of primary osteoblast lineage cells to glutamate caused a dose-dependent increase in the expression of ALP (**a**), while the number of viable cells was decreased with the increasing glutamate concentrations (**b**). Values are expressed as mean ± SE ($n = 6$; $^{\#}p < 0.05$ vs. control). Treatment of primary osteoblast lineage cells by the GluR agonist SOS (**c**, **d**) led to the dose-dependent increase in ALP (**c**), and a decrease in the number of viable cells (**d**). Values are expressed as mean ± SE ($n = 6$; $^{\#}p < 0.05$ vs. control)

activate the osteoblastic GSMS, subsequently leading to cellular differentiation and MAPK-related signal cascades.

Blocking glutamate uptake using nonselective inhibitors of GluT increased extracellular glutamate and promoted osteoblast differentiation in a dose-dependent manner. The use of selective inhibitors for specific GluTs did not result in such an effect. The expression of at least five GluTs by osteoblasts suggests that blocking one specific transporter, as is the case for DHK, an inhibitor of Slc1a2 [29], is not effective. DHK was shown to be inefficient in inducing a change in extracellular glutamate concentration and in promoting osteoblast differentiation. Previous studies reported the release of glutamate by osteoblast lineage cells [30–32], and these findings were confirmed in the current study. Furthermore, the data of this study demonstrate that re-uptake of glutamate by GluT negatively modulates glutamate-stimulated osteoblastic differentiation. Osteoblas tic and osteocytic glutamate were previously suggested to take part in the control of bone remodeling on the basis of the previous findings that mechanical load controls the expression of GluT and GluR in bone [14, 15, 33]. Together with the data presented in this study, it is suggested that glutamate signaling in osteoblast lineage cells might be involved in auto-/paracrine control of bone modeling and remodeling in response to mechanical stimulation.

The data in this study correspond to previous findings that glutamate supports the development of osteoblast lineage cells [34, 35]. The expression of GluR, both ionotropic and metabotropic, by primary osteoblasts indicates that AAs play a role in bone homeostasis. While glutamate is a well-characterized neurotransmitter in the CNS, recent studies have demonstrated that nonexcitable peripheral cells, such as megakaryocytes and tumor cells, express GluR and are responsive to AAs [36–39]. Furthermore, other studies indicate that nerve fibers on the bone surface generate glutamate signals [33, 40, 41]. In the current study, however, we emphasize that glutamate can be released by bone cells and that it might be possible for AAs to act through an autocrine mechanism.

The GluR on osteoblast lineage cells has been shown to transduce a glutamate signal. Exposure of the cells to the GluR agonists L-HCSA and L-CSA caused a significant increase in ALP activity, and the GluT inhibitor SOS was also found to exert agonistic effects through GluR [42–44].

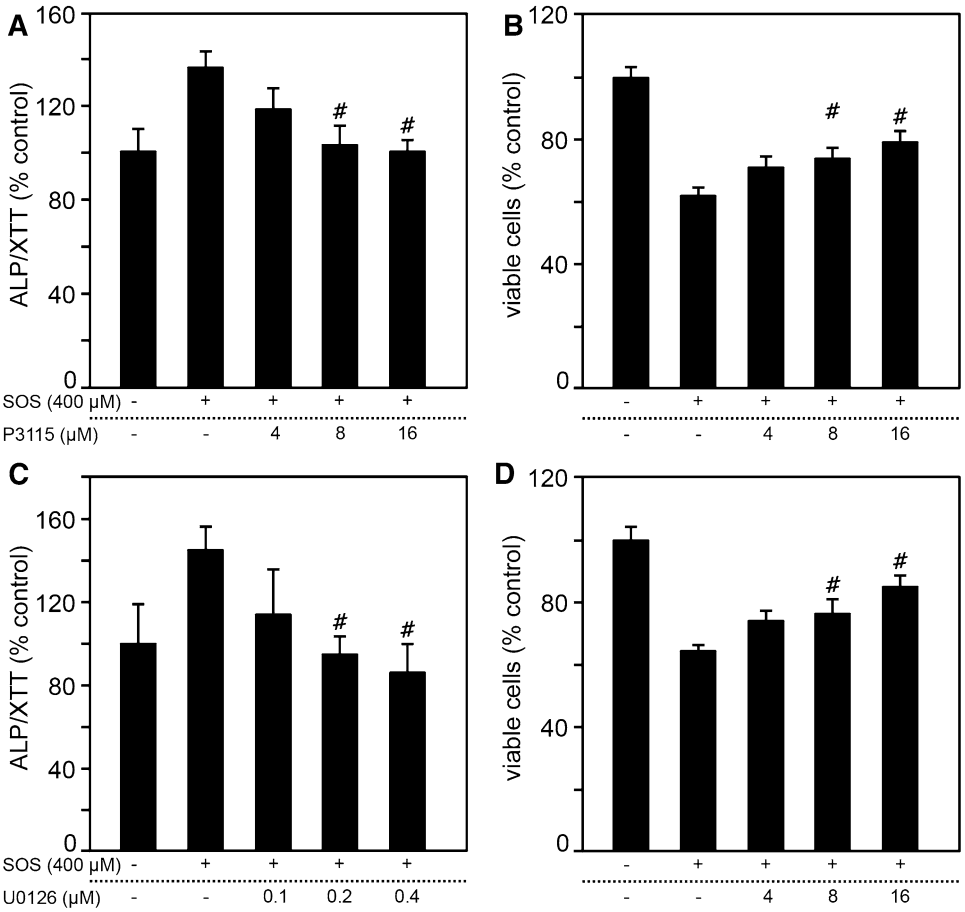

Fig. 7 PKC and Erk1/2 mediate the effects of glutamate on osteoblast function. Treatment of primary osteoblast lineage cells with either PKC inhibitor P3115 (**a**,**b**) and Erk1/2 inhibitor U0126 (**c**,**d**), respectively, reversed the effects of the GluT antagonist and GluR agonist SOS on osteoblast differentiation and proliferation. Values are expressed as mean ± SE ($n = 6$; $^{\#}p < 0.05$ vs. treatment with SOS)

In the absence of extracellular glutamate, SOS treatment led to an increase in osteoblast differentiation while decreasing cell proliferation. Thus, SOS exerts dual functions in the glutamate signaling system, acting as both a GluT antagonist and a GluR agonist.

Glutamate signaling in post-synaptic neurons induces Ca^{2+} and Na^{+} influx into the cells and signals through G-protein coupled receptors, inducing a multitude of biological processes, including cell proliferation, differentiation, and apoptosis [45, 46]. In the cells of the CNS, glutamate signaling cascades are well known to be associated with CaMKII, PKC, PI3 K, and P38 activations [23, 47]. Glutamate signal pathways in cancer cells have been showed to active RAS [48, 49]. A study showed that NMDA receptor activation increases PKA, PKC, and PI3 K protein levels responsible for rat osteoblast differentiation. Previous studies have indicated that glutamate signals in osteoblastic cell lines lead to the activation of Wnt and AP-1 pathways [50, 51]. In the current study, Erk1/2 and PKC were activated within 30 min and in sequence, demonstrating that the MAPK signal pathway is involved in glutamate signaling and leads to osteoblast differentiation.

In summary, the current data have identified a GSMS in primary murine osteoblasts, demonstrated functions of glutamate, GluR, and GluT during osteoblast development and investigated their activity in osteoblast lineage cells. The stimulation of a signaling cascade by glutamate, either through the inhibition of GluT or through the activation of GluR, triggers activation of MAPK pathways through PKC and ERK1/2, eventually leading to osteoblast differentiation. The release of glutamate by osteoblast lineage cells could contribute to the capacity of osteocytes acting as mechanosensors, thereby providing an autocrine mechanism that leads to an anabolic response after mechanical stimulation of bone. The use of glutamate as a therapeutic agent, however, remains to be investigated. Two major aspects may be crucial in the

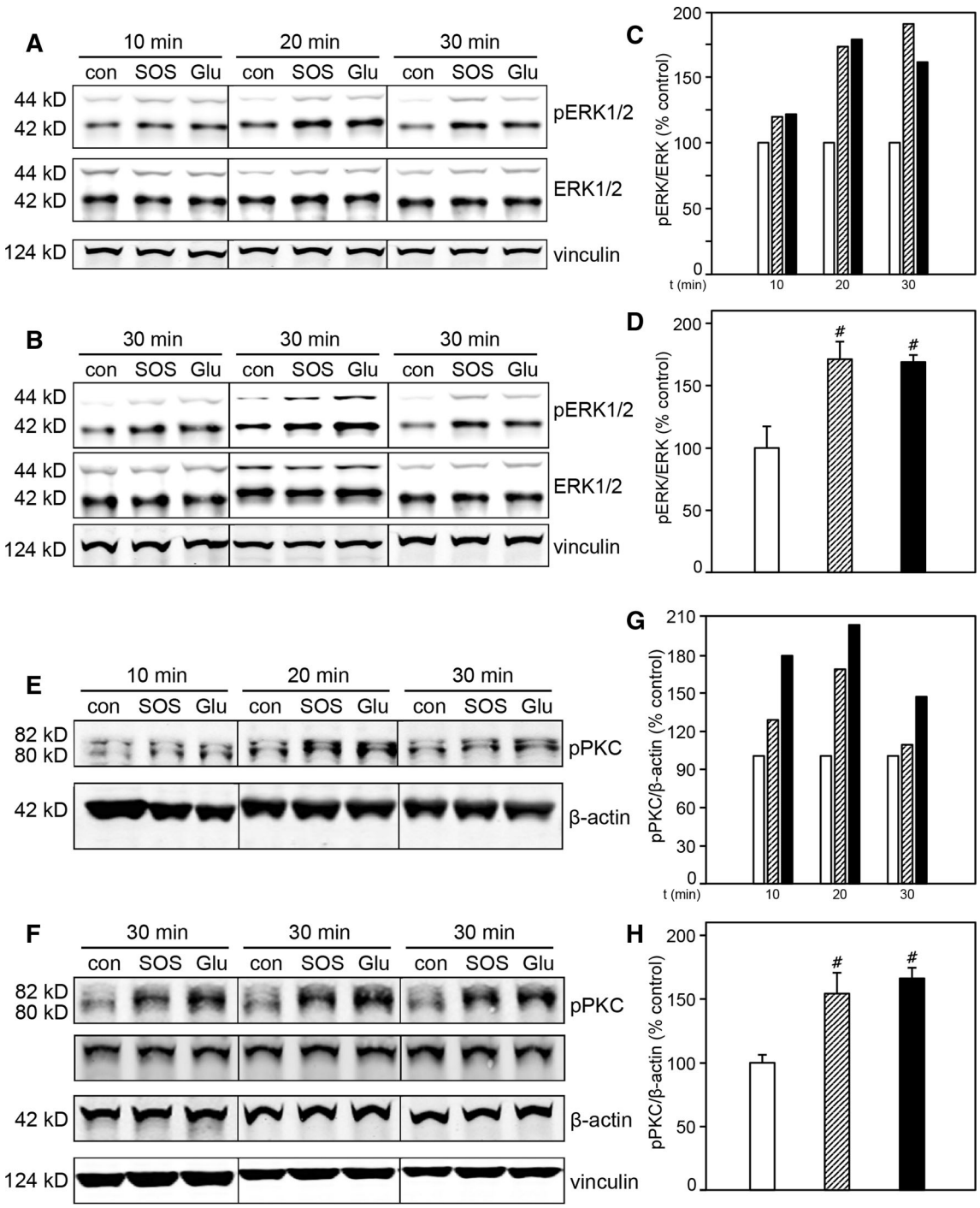

Fig. 8 SOS and glutamate induce signaling cascades involving PKC and Erk1/2 in osteoblasts. Exposure of primary osteoblast lineage cells with glutamate and SOS, respectively, leads to phosphorylation of Erk1/2 (**a**,**b**) and PKC (**c**,**d**) within 30 min. Values are expressed as mean ± SE ($n = 3$; $^{\#}p < 0.05$ vs. control)

application of glutamate in patients, (i) to reach the adequate concentrations at the adequate sites, and (ii) to restrict the distribution of glutamate to the sites where an anabolic effect is sought after.

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Compliance with Ethical Standards

Conflict of Interest Wenjie Xie, Silvia Dolder, Mark Siegrist, Antoinette Wetterwald, and Willy Hofstetter declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent All animals used in this study were housed in the Central Animal Facility at the Medical Faculty of the University of Bern, complying with the Swiss Guidelines for Care and Use of Experimental Animals. The experiments described in this study were approved by the State Committee for the Control of Animal Experimentation (permit number BE23/13 to WH).

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