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Original Article

Hylonome

Regulation of the mGluR5, EAAT1 and GS expression by glucocorticoids in MG-63 osteoblast-like osteosarcoma cells

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Abstract

Introduction: Growth factors, cytokines, sex steroid hormones and glucocorticoids have differential and complex effects on skeletal metabolism. Recently, the presence of the glutamatergic (Glu) system in bone cells has provided new evidence for its possible role in bone physiology. Consequently, we have investigated the regulation of certain components of the Glu system by glucocorticoids in MG-63 osteoblast-like osteosarcoma cells, *in vitro*. Materials and Methods: We characterized the effects of dexamethasone on the expression of the mGluR5, EAAT1 and GS, at mRNA and protein level, using relative quantitative RT-PCR and Western blot analysis, respectively. Results: We confirmed the induction of GS expression by dexamethasone published previously. In addition, we documented for the first time the expression of the mGluR5 and EAAT1 in MG-63 cells, as well as the ability of dexamethasone to upregulate the expression of the mGluR5 and EAAT1 in the MG-63 cells. Conclusions: Components of the glutamatergic system may play a role in bone pathophysiology.

Keywords: MG-63 cells, mGluR5, EAAT1, GS, Dexamethasone

Introduction

The proliferation, development and differentiation of bone cells are controlled by growth factors, cytokines and steroid hormones, some of which are synthesized and/or activated in bone microenvironment[1]. The glucocorticoid effects on bone tissue are fairly complex. Glucocorticoids act mainly via a nuclear receptor (GR) which has two isoforms, GRα and GRβ. In humans, the GRα is found in osteoblasts and osteocytes, however, it has not been detected in osteoclasts[2]. Recently, the GRβ has been detected in osteoclasts[3]. GR signaling is required for normal bone formation, *in vivo*, and synthetic glucocorticoids, such as dexamethasone, were shown to promote osteoblasts' differentiation in several *in vitro* and *in vivo* models. The mechanism by which glucocorticoids affect bone tissue is complex, which includes regulation of proliferation/apoptosis of osteoblasts and osteoclasts[4,5], as well as the modulation of the RANK/RANKL/OPG system, the main regulatory system of osteoclastogenesis[6]. Since glucocorticoids are widely used to treat a variety of chronic diseases, including collagen diseases and skin disorders[7], long-term glucocorticoid therapy was documented to induce bone loss, which is the most common cause of secondary osteoporosis[8].

Recently, it has been demonstrated that the existence of another bioregulatory system in bone, namely the glutamatergic system (Glu system)[9-11]. Components of the Glu system are the Glu receptors (GluRs) and the Glu transporters (GluTs). Glutamate is a well-known neurotransmitter in the mammalian central nervous system (CNS)[12]. The GluRs are divided into the ionotropic GluRs (iGluRs), which are directly gated ion channels and are sub-divided into three groups (NMDAR, AMPAR and KARs) and the metabotropic GluRs (mGluRs) that are coupled with a G protein and are also sub-divided into three groups (group I, II and III)[13,14]. Group I mGluRs (mGluR1 and mGluR5) are coupled to phospholipase C (PLC), while group II (mGluR2 and mGluR3) and III (mGluR4, mGluR6, mGluR7 and mGluR8) are negatively coupled to adenyl cyclase[15-17].

Apart from the GluRs, which are responsible for signal input, there are two Glu transport systems, the vesicular GluTs (VGluTs: VGluT1-3), which are responsible for signal

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Abbreviations used:

NMDA, N-methyl-D-aspartate
NMDAR, NMDA Receptor
AMPA, $_{DL}$-a-amino-3-hydroxy-5-methylisoxasole-4-propionate
AMPAR, AMPA Receptor
KAR, Kainate Receptor
EAAT, excitatory amino acid transporters
GLAST, glutamate-aspartate transporter
DHPG, (RS)-3,5-dihydroxyphenylglycine
IP3, inositol-1,4,5 triphosphate
DAG, diacylglycerol
mTOR, mammalian target of Rapamycin
PI3K, phosphatidylinositol-3-kinase
GS, glutamine synthetase

output through exocytic release[18] and the plasma GluTs for signal termination[19]. The excitatory amino acid transporter family (EAAT: EAAT1-5) is found in excitatory synapses of human CNS[20]. Because of the very low permeability of the blood-brain barrier to glutamate, the brain must have the ability to synthesize and recycle this molecule. One important enzyme that is playing a role in the metabolic pathway of Glu is the glutamine synthetase (GS). GS is located in astrocytes and is responsible for the conversion of Glu to glutamine, which in turn is taken up by neurons and this pathway is called glutamate-glutamine cycle[21-23].

In a previous study, we have characterized the expression of components for the Glu system in the human MG-63 osteoblast-like osteosarcoma cell line[24]. The aim of this study was to investigate the regulation of certain components of the Glu system by glucocorticoids in the MG-63 osteoblast-like osteosarcoma cells.

Materials and methods

Cell culture

The human MG-63 osteosarcoma cell line was obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA). Cells were grown in 75-cm^2 culture flasks and maintained in a 5% CO_2 incubator at 37°C. The culture medium was the RPMI (Gibco #61870-010), containing 10% fetal bovine serum (FBS) (Gibco #310108-165), 100 μU/ml, 100 μg/ml Penicillin/Streptomycin (Invitrogen #15070-063), 2 mM L-glutamine (Invitrogen #25030-081) and 10 mM Hepes (Invitrogen #15630-080).

For RNA extraction, MG-63 cells were grown in 75-cm^2 culture flasks. For protein extraction, the MG-63 cells were subcultured into 6-well plates and cultured with RPMI medium containing 10% FBS. At 70% confluence, the cells were incubated in RPMI medium containing 0.5% FBS to eliminate the effects of endogenous factors. Under these conditions, MG-63 cells were exposed to dexamethasone (100 nM) for 48 hours.

Relative Quantitative RT-PCR

MG-63 cells were detached with Trypsin/EDTA solution (Gibco #25200-056) and then collected by centrifugation. For RT-PCR, total RNA was isolated from MG-63 cells using the Trizol reagent (Invitrogen #15596-026). First strand cDNA was synthesized from 1.5 μg of RNA mixed with 10 mM dNTPs (Invitrogen #10297018), 3 μg/μl Random Hexamer Primers (Invitrogen #48190-011) and filled up to 12 μl with depc-treated ddH_2O. The reaction was then heated to 65°C for 5 minutes and quick-chilled on ice water. The RT buffer containing 200 U/μl of Superscript II RNase H- Reverse Transcriptase (Invitrogen #18064-014) was then added and the reactants were incubated at 42°C for 50 minutes and 70°C for 20 minutes.

The cDNA was amplified by relative quantitative RT-PCR with specific primers for mGluR5 and EAAT1 selected using the Primerfinder Program based on sequences obtained from the gene bank, whereas the primers for GS were previously published pair[22,25]. 18S rRNA was used as an internal standard to normalize the mRNA expression.

The following primers were used:
mGluR5 primers [Human mGluR5 (NM_000842)]:
Forward primer: 5′-TCCAGAATTTGCTCCAGCTT-3′
Reverse primer: 5′-CTTCCATCCCACTTTCTCCA-3′

EAAT1 primers [Human EAAT1 (D26443)]
Forward primer: 5′-GGAAGGGCACAAAGGAAA-3′
Reverse primer: 5′-CCCCCCAATCACACCCAT-3′

GS primers [Human GS (BC031964)]
Forward primer: 5′-TACATCGAGGAGGCCATTGA-3′
Reverse primer: 5′-AGCTGGAGGTCTAGTCCACT-3′

The PCR mix for the amplification of GS was carried out at 25 μl, consisting of 5 U/μl Taq DNA Polymerase recombinant (Invitrogen #10342-020), 10X PCR Buffer, 10 mM of each dNTP, 50 mM $MgCl_2$, 2 μl 18S Primer:Competimer mixture (Classic 18S Internal Standards, Ambion #1716) in a ratio 1:9 and 1 μl cDNA. The amplification of EAAT1 was carried out at 25 μl, consisting of Platinum PCR supermix (Invitrogen #11306-016), with the addition of 1 μl 18S Primer: Competimer mixture (Universal 18S Internal Standards, Ambion #1718) in a ratio 1:9 and 2 μl cDNA, while the amplification of mGluR5 consisted of Platinum PCR supermix, with 2 μl 18S Primer: Competimer mixture (Universal18S Internal Standards, Ambion #1718) in a ratio 1:17 and 1 μl cDNA.

Primer set for mGluR5 was amplified at 94°C for 30 seconds, 60°C for 15 seconds and 68°C for 15 seconds (43 amplification cycles), for EAAT1 was amplified at 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 15 seconds (38 amplification cycles), while the program for GS PCR was 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds (27 amplification cycles). Amplification products were separated by agarose gel (1.8%), visualized with ethidium

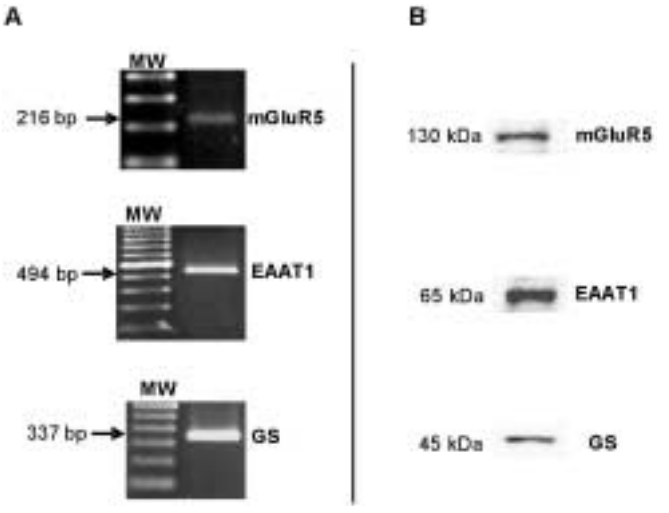

Figure 1. Representative RT-PCR and Western blot analysis of mGluR5, EAAT1 and GS in MG-63 osteoblast-cells. mGluR5, EAAT1 and GS mRNAs (**Panel A**) and proteins (**Panel B**) are expressed in MG-63 cells. Marker 100 bp DNA ladder (Invitrogen #15628-050) and PageRuler Prestained Protein Ladder (Fermentas #SM0671) were used for the approximation of molecular weight of PCR products and blotted proteins, respectively.

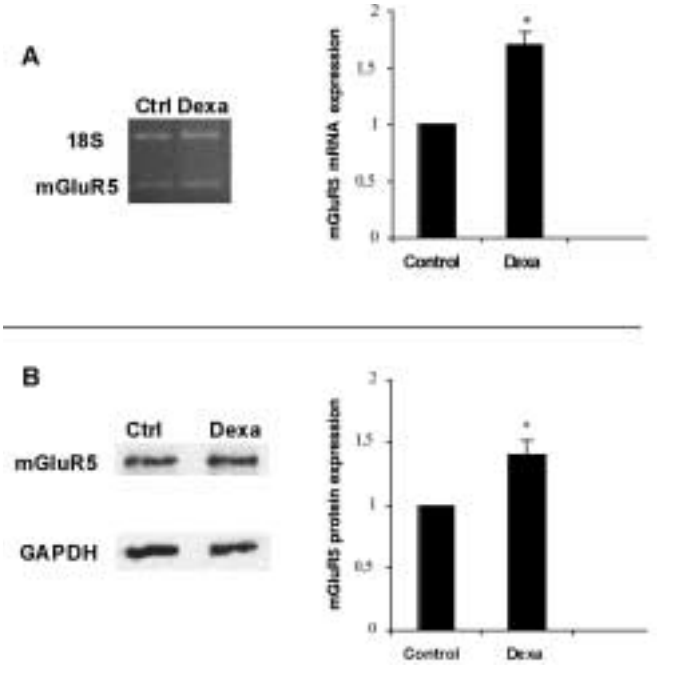

Figure 2. Effect of dexamethasone on mGluR5 expression in MG-63 osteoblast cells. There was a little increase in mGluR5 mRNA and protein levels, in MG-63 cells treated with dexamethasone (Dexa, 100nM) for 48 h. Representative images of mGluR5 and house keeping genes, 18S and GAPDH, from relative quantitative RT-PCR (**Panel A**) and Western blot analysis (**Panel B**) are shown. Relative quantitative RT-PCR (**Panel A**) and Western blot analysis (**Panel B**) are represented also as mRNA and protein expression, respectively with means ± SE (n=3). Statistical analysis was performed by Student's T-test. In each analysis, p values reached the most significant level ($p<0.05$).

bromide and photographed (Kodak DC290 camera). The quantifications of PCR products were performed with Kodak EDAS 290 program.

Western blot analysis

After the exposure to dexamethasone, MG-63 cells were detached with a cell scraper and then collected by centrifugation. Pellet was lysed in Triton-X-100-based lysis buffer (20 mM Tris-HCl, pH:7.4, 1% Triton X-100, 1 mM EDTA, 5 mM DTT, 150 mM NaCl) supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4). After the centrifugation, the protein was in the solution, which is transferred and stored at -80°C for protein analysis. The protein content was determined by using the bradford protein assay. Forty μg samples of total protein were resolved under reducing conditions by 7% SDS-PAGE for the detection of mGluR5 protein and 10% SDS-PAGE for EATT1 and GS proteins. The gels were transferred onto nitrocellulose transfer membrane. After blocking with 5% non-fat dry milk, the membranes were incubated overnight at 4°C, with mGluR5 rabbit polyclonal antibody (Upstate #06-451) at a 1:500 dilution or EAAT1 rabbit polyclonal antibody (Santa Cruz Biotechnology #sc-15316) at a 1:500 dilution or GS rabbit polyclonal antibody (Santa Cruz Biotechnology #sc-9067) at 1:1000 dilution in TBS-Tween containing 1% non-fat dry milk. Thereinafter, the membranes were incubated with goat HRP conjugated antirabbit IgG (1:2000, Santa Cruz Biotechnology #sc-2004) for 1 h. The immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce #34080). Expression of GAPDH (1:2000, Santa Cruz Biotechnology #sc-32233), as reference protein, was also analysed in the same proteins extracts. The quantifications of proteins were performed with Kodak EDAS 290 program.

Results

Expression of mGluR5, EAAT1 and GS in MG-63 osteoblast cells

In our previous study, we documented the presence of mGluR5 (216 bp), EAAT1 (494 bp) and GS (337 bp) at mRNAs level in MG-63 cells[24]. Herein, we have reconfirmed these data for the mGluR5, EAAT1 and GS mRNA expression and we report their expression at protein level (for mGluR5 and EAAT1 for the first time) as bands of approximately 130 kDa, 65 kDa and 45 kDa by Western blot analysis, respectively (Figure 1).

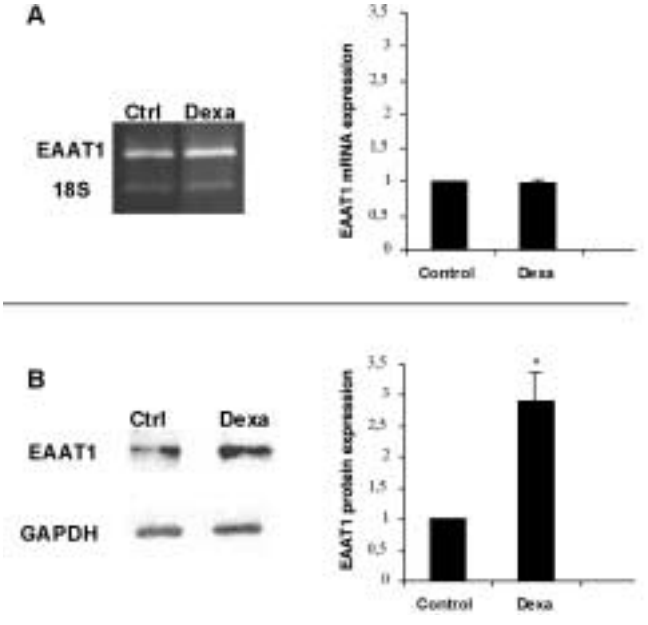

Figure 3. Effect of dexamethasone on EAAT1 expression in MG-63 osteoblast cells. In contrast to mRNA expression, EAAT1 protein was increased by dexamethasone (Dexa, 100nM) within 48 h. Representative images of EAAT1 and house keeping genes, 18S and GAPDH, from relative quantitative RT-PCR (**Panel A**) and Western blot analysis (**Panel B**) are shown. Relative quantitative RT-PCR (**Panel A**) and Western blot analysis (**Panel B**) are represented also as mRNA and protein expression, respectively with means ± SE (n=3). Statistical analysis was performed by Student's T-test ($p<0.05$ for Western blot analysis).

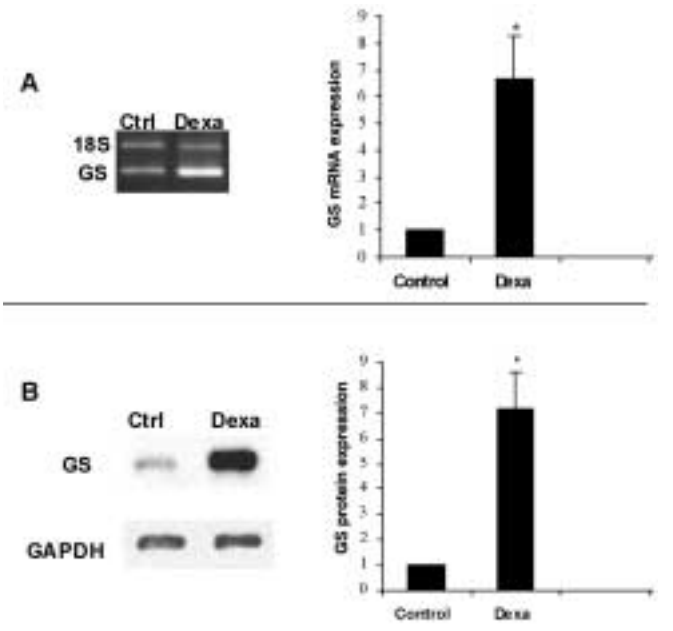

Figure 4. Effect of dexamethasone on GS expression in MG-63 osteoblast cells. Both GS mRNA and protein were significantly increased in MG-63 cells treated with dexamethasone (Dexa, 100nM) for 48 h. Representative images of GS and house keeping genes, 18S and GAPDH, from relative quantitative RT-PCR (**Panel A**) and Western blot analysis (**Panel B**) are shown. Relative quantitative RT-PCR (**Panel A**) and Western blot analysis (**Panel B**) are represented also as mRNA and protein expression, respectively, with means ± SE (n=3). Statistical analysis was performed by Student's T-test ($p<0.05$).

Regulation of mGluR5 by dexamethasone in MG-63 osteoblast cells

The regulation of mGluR5 expression in MG-63 cells, after exogenous administration of the dexamethasone was analyzed by relative quantitative RT-PCR and Western blot analysis. Under these experimental conditions, mGluR5 was upregulated at both the mRNA levels (mean: 1.7-fold; 1.9, 1.5, 1.7) and protein levels (mean: 1.4-fold; 1.6, 1.5, 1.2). Each analysis was repeated independently at least three times (Figure 2).

Regulation of EAAT1 by dexamethasone in MG-63 osteoblast cells

Treatment of MG-63 cells with dexamethasone did not show any significant difference in EAAT1 mRNA expression, whereas we observed an induction of EAAT1 protein. This increase was estimated to be 2.8-fold (mean: 2.8-fold; 3.0, 2.0, 3.5) compared to the control expression level in MG-63 cells. RT-PCR and Western blot analysis was repeated independently at least three times (Figure 3).

Regulation of GS by dexamethasone in MG-63 osteoblast cells

Dexamethasone upregulated the mRNA and protein expression level of GS, which were estimated to be 6.7-fold (mean 6.7-fold; 9.9, 5.3, 5.0) and 7.2-fold (mean: 7.2-fold; 9.2, 8.0, 4.4) higher than in controls, respectively. Each experiment was repeated independently at least three times (Figure 4).

Discussion

Recently, it has been detected the expression of the glutamatergic system outside the CNS[9,10]. This peripheral expression of Glu system includes not only normal, but also cancer cells. Functional molecules of the Glu system have been identified in pinealocytes, pancreas, megakaryocytes, heart, keratinocytes, melanocytes, T leukemia cells, thyroid carcinoma, melanoma, prostate, kidney, lung and several other normal or cancer tissues and organs[26]. In bone tissue, the first evidence for the presence of Glu system in bone cells was by the documentation of GLAST-1/EAAT1 transporter after mechanical loading on rat osteocytes[11]. Thereafter,

functional Glu receptors (GluRs) and transporters (GluTs) were detected in osteocytes, osteoclasts and osteoblasts[27].

GluRs and GluTs are the main components of the Glu system. GluRs (iGluRs and mGluRs) subject the effect of Glu and transmit this action either by gated ion channels (iGluRs) or by mGluRs, which activate intracellular signaling pathways[13,14]. EAATs are the human glutamate transporters that are responsible for the extracellular Glu level[19], whereas enzymes such as GS, which metabolize Glu to glutamine, is responsible for the control of the intracellular Glu concentration[21].

In our previous study, we characterized the Glu system in human MG-63 osteoblast-like osteosarcoma cells. In particular, we confirmed the expression of NR1, NR2A, NR2B, NR2D (sub-units of NMDAR) and GS mRNAs in MG-63 cells. In addition, we documented for the first time the mRNA expression of the NR3A sub-unit of NMDAR, this of EAAT1 and of mGluR1, mGluR2, mGluR3, mGluR4, mGluR5 and mGluR8 mRNAs in MG-63 cells[24]. These detections enable us to use the MG-63 cell line as a model for the study of the regulation of the Glu system in bone pathophysiology.

In this study, we have investigated the relationship between the administration of glucocorticoids and the expression of elements of the Glu system. Our preference of mGluR5, from the other mGluRs which were detected in MG-63 cells, was based upon the nature of its signal transduction pathway. Group I mGluRs (mGluR1 and mGluR5) are coupled to PLC, causing the formation of IP_3 and DAG, which in turn produce increases in cytosolic free Ca^{2+} and activation of protein kinase C[16]. The interesting thing is that another intracellular signal transduction pathway of group I mGluRs has been detected. In particular, it was shown that activation of group I mGluRs (mGluR1 and mGluR5) with DHPG, an agonist that activates the group I, can trigger the activation of the PI3K-Akt-mTOR signaling pathway in the mouse hippocampal area. These three signaling molecules regulate translation initiation, which means that this cascade couples group I mGluRs to the protein translation machinery[28].

Since the mGluR1 was not detected at protein level in MG-63 cells (data not shown), we have focused our investigation on the expression of mGluR5, EAAT1 and GS in MG-63 cells and their regulation by dexamethasone both at the mRNA and protein level. According to the literature, dexamethasone is reported to regulate GS activity in muscle cells[29,30], in 3T3-L1 adipocytes[31], in astrocytes[32,33], in C6-glioma cells[34,35] and in osteoblasts[25]. In addition, the effect of dexamethasone on EAAT1 is about the mechanism of dexamethasone on inhibiting the downregulation of GluTs like GLAST/EAAT1, GLT-1 and EAAC1, at least in part, after the increasing of synaptic excitatory amino acids concentration in morphine-tolerant rats after morphine challenge[36]. Less information exists about mGluR5 expression and the regulation of other components of Glu system by dexamethasone in osteoblast-like cells.

Herein, we confirmed the expression and the induction of GS by dexamethasone in MG-63 cells[25]. Moreover, we report for the first time the glucocorticoid effects on the upregulation of mGluR5 at transcription and protein level and the significant upregulation of EAAT1 at protein levels but not at transcription levels. These data suggest that glucocorticoids may stabilize EAAT1 mRNA rather than upregulating EAAT1 transcription.

Conclusion

Our data have presented strong evidence for the presence of components of the Glu system in MG-63 osteoblast-like cells. Our data suggest that some of the components of the Glu system are possibly implicated in bone pathophysiology.

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