The Glutamatergic System Expression in Human PC-3 and LNCaP Prostate Cancer Cells

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Abstract. *Background: The glutamatergic system (Glu system) comprises the Glu receptors (GluRs), the Glu transporters (GluTs) and glutamine synthetase (GS). Materials and Methods: Using PCR-based detection and Western blot analysis, the expression of Glu system components was assessed in human androgen-independent PC-3 and androgen-dependent LNCaP prostate cancer cells. Results: iGluRs, such as NR1, NR2A, NR2C, NR2D and NR3B; mGLuRs such as mGluR1, mGluR2, mGluR3, mGluR4 and mGluR5; GluTs such as EAAT1, EAAT2, EAAT3 and EAAT5; and GS mRNA were steadily expressed in both cell lines. In addition, NR3A, mGluR6, mGluR8 and EAAT4 mRNA were differentially expressed in PC-3 and LNCaP cells. mGluR7 and EAAT4 mRNA expression was induced and mGluR8 was silenced by dihydrotestosterone (DHT) treatment in LNCaP cells. GS, EAAT1 and mGLuR5 were also detected at the protein level in both PC-3 and LNCAP cells. Conclusion: These data suggest that the Glu system could be an important regulator of prostate cancer cell biology.*

Glutamate (Glu) is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) (1). The glutamatergic system (Glu system) comprises Glu receptors (GluRs) and Glu transporters (GluTs). The GluRs, which are responsible for signal input, are divided into two categories: the ionotropic GluRs (iGluRs) and metabotropic GluRs (mGluRs) (2, 3). The iGluRs are directly gated ion channels having only excitatory action (2), while mGluRs promote intracellular signal transduction pathways *via* activation of G proteins (4, 5). The GluTs are divided into two categories, the plasma GluTs that are responsible for signal termination (6) and the vesicular GluTs (VGluTs) responsible for signal output through exocytic release in the CNS (7). Finally, the metabolism of Glu by glutamine synthetase (GS) completes the Glu system, controlling intracellular glutamate signaling pathways by the conversion of glutamate to glutamine (8-10).

The iGluRs are subdivided into three groups, *N*-methyl-D-aspartate (NMDA), a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate (KA) receptors, based on their pharmacology and structural similarities, and are named according to the type of synthetic agonist that activates them (11). NMDA receptor (NMDAR) is a hetero-oligomeric protein composed of two classes of subunits: two NR1 subunits and two or three NR2 (NR2A, NR2B, NR2C and NR2D) subunits that co-assemble to form a tetramer (12) or pentamer (13). There is a third subunit, NR3 (NR3A and NR3B), which is a regulatory subunit that decreases the NMDA channel activity (14). NMDAR is highly permeable to Ca^{2+}, with sensitivity to blockade by Mg^{2+} in a voltage-dependent manner (15, 16).

The mGluRs are G-protein-coupled receptors (GPCRs) that have been subdivided into three groups, group I, II and III, based on sequence similarity, pharmacology and intracellular signaling mechanisms. The mGluRs possess a seven-transmembrane domain with an extracellular *N*-terminus and intracellular *C*-terminus domain. Group I mGluRs (mGluRl, mGluR2, mGluR3, mGluR4 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 (cAMP). These eight mGluRs are products from different genes (4, 5, 17).

The Glu system, as the main excitatory neurotransmission system, plays a key role in neuronal progress; however, various characteristics of neuronal embryonic cells are also shared by tumour cells (18). There is increasing evidence to suggest that the Glu system may be involved in cancer biology (19). Herein, we have characterized for the first time the expression of components of the Glu system in two human prostate cancer cell lines, namely the PC-3 androgen-independent and LNCaP androgen-dependent cells.

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Materials and Methods

Cell culture. The PC-3 androgen-insensitive cell line and the LNCaP androgen-sensitive cell line were obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA). PC-3 cells were maintained in Dulbecco's modified Eagle's medium/F-12 (Cambrex, Walkerville, MD USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 100 U/ml penicillin/streptomycin (Cambrex) at 37˚C in a humidified atmosphere of 5% CO_2. LNCaP cells were maintained in DMEM/F-12 (Cambrex) supplemented with 10% FBS (Biochrom), 100 U/ml penicillin/streptomycin (Cambrex) and 100 nM dihydrotestosterone (DHT, Sigma-Aldrich, MO, USA) in a humidified atmosphere of 5% CO_2 at 37˚C.

Isolation of RNA and PCR conditions. RNA was isolated using TRI-Reagent (MRC, Cincinnati, OH USA # RT-111). The RNA was diluted in sterilized nuclease-free water (QIAGEN# 129117). The reverse transcription (RT) procedure was performed using Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase, RNase H^- (FINZZYMES# F-572L) and Pre-Mixed dNTPs (10 mM each) (HT BIOTECHNOLOGY# SB-25U). Two different RT reactions (2 μg final RNA concentration) were performed for each cell line, one using oligo dT_{18} (Fermentas# SO132) and the other using a mix of Random Hexamer primers (Fermentas #SO142) and OligodT_{18}. The reactions were then heated to 70˚C for 5 min and quick-chilled on iced water. The RT buffer containing 200 U/μl of M-MuLV reverse transcriptase was then added and the reactants were incubated at 37˚C for 60 min. The M-MuLV was inactivated at 70˚C for 5 min. The PCR reactions were performed using Taq PCR MasterMIX kit (QIAGEN#201445). Each reaction comprised a 45 cycle program (of 1 min annealing time). In the case of mGluR3 and mGluR8, a 35-cycle program was performed. DNA extracted from leukocytes, PC-3 and LNCaP cells, served as negative controls. The primers used in the reactions were generated using the FastPCR program (20) based on sequences obtained from Genebank, except for mGluR3, mGluR6, mGluR8, and NR3B, which have been previously published (21). The primer sequence and the expected PCR product size along with the annealing temperature for each gene are presented in Table I. The PCR amplified fragments (amplicons) were subjected to endonuclease digestion as presented in Table II. The digestion products were separated on 3% agarose gels and the fragmented bands were visualized by ethidium bromide staining. In each case, the endonuclease digestion confirmed the identity of the amplicons.

Western blot analysis. PC-3 cells were seeded in p100 plates and grown in DMEM/F-12 containing 10% FBS. Twenty-four hours prior to protein extraction, the growth medium was changed to 0.5% FBS. Cells were then detached from the plates with a cell scraper and then collected by centrifugation. The 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 dithiothreitol (DTT), 150 mM NaCl) supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM NaF, 1 mM Na_3VO_4). After the centrifugation, the final protein solution was transferred and stored at –80˚C for protein analysis. The protein content was determined using the Bradford protein assay. Samples of total protein (40 μg) were resolved under reducing conditions by 7% SDS polyacrylamide gel electrophoresis (SDS-PAGE) for the detection of mGluR5 protein and 10% SDS-PAGE for EAAT1 and GS proteins. The gels were transferred onto nitrocellulose transfer membranes. After blocking with 5% nonfat dry milk, the membranes were incubated overnight at 4˚C, with mGluR5 rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA; #06-451) at a 1:500 dilution or EAAT1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA USA #sc-15316) at a 1:500 dilution or GS rabbit polyclonal antibody (Santa Cruz Biotechnology #sc-9067) at 1:1,000 dilution in TBS-Tween containing 1% nonfat dry milk. Thereafter, the membranes were incubated with goat horseradish peroxidase (HRP)-conjugated antirabbit IgG (1:2,000, Santa Cruz Biotechnology #sc-2004) for 1 h. The immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, Illinois, USA; #34080). Expression of GAPDH (1:2,000, Santa Cruz Biotechnology #sc-32233), as reference protein, was also analyzed in the same protein extracts.

Results

Using our specific primers for the detection of the mRNA of the Glu system components and under our PCR conditions (Tables I and II), we have identified certain components of the Glu system in human PC-3 and LNCaP prostate cancer cells.

Certain components of the Glu system were steadily expressed at the mRNA level in both the androgen-independent PC-3 and androgen-dependent LNCaP cells. These included expression of iGluRs such as NR1, NR2A, NR2C, NR2D and NR3B; mGLuRs such as mGluR1, mGluR2, mGluR3, mGluR4 and mGluR5; GluTs such as EAAT1, EAAT2, EAAT3 and EAAT5; and GS (Table III).

In addition, NR3A, mGluR6, mGluR8, and EAAT4 were differentially expressed in the androgen-independent PC-3 and androgen-sensitive LNCaP cells (Table III). Interestingly, the expression of *mGluR7* and *EAAT4* mRNA was induced, whereas that of *mGluR8* was silenced by DHT treatment in LNCaP cells. These data suggest that these components of the Glu system are hormonally regulated in human LNCaP prostate cancer cells.

We proceeded to the detection of the expression of EAAT1, GS and mGluR5 at the protein level, all of which are known to be important components of the Glu system in other malignant cell lines (22). All of them were expressed in PC-3 and LNCaP cells (Figure 1).

Discussion

There is new evidence to suggest that the Glu system may be involved in cancer biology (19). Herein, we documented that all the necessary elements of an active Glu system (GluRs, GluTs and GS) are expressed in human prostate cancer cell lines. Therefore, it is possible that PC-3 and LNCaP prostate cancer cells possess ion-dependent channel activity and intracellular G protein-dependent signaling pathways controlled by glutamate.

Table I. *PCR primers and PCR conditions for the detection of Glu System components in PC-3 and LNCAP cells.*

iGluRs	Annealing temperature (˚C) (Amplicon size, bp)	mGluRs	Annealing temperature (˚C) (Amplicon size, bp)
NR1		mGluR.1	
F-GCTGATGTCTTCCAAGTATGCGGA	60	F-GCTGCAGAACATGCACCATGC	62
R-ATCACTCAGCGTGGGCTTGACGTA	(280)	R-TCATGCCAGGTTCCAACGTGCA	(237)
NR2A		mGluR.2	
F-CTCTCCTTGGAAGAGGCAGATCGA	58	F-CTGGTTCCGTGAATTCTGGGAGCA	60
R-CAGCTTCCAGTAGAAGAGGTGCTC	(232)	R-AAGCGGACCTCATTGTGGGTGTC	(300)
NR2B		mGluR.3	
F-TAGCTGCCTTCATGATCCAAGAGG	Not detected	F-AGCCATCACCAGTCATCTGT	60
R-CCAGTGGAAGCAAAGACCTTCCCA	(334)	R-ATGAGCCACACAGACACCAT	(220)
NR2C		mGluR.4	
F-TGCATCGACATCCTCAAGAAGCTG	62	F-TGCGCAACGATTCTGCCGAGTA	60
R-ACTTGCCGATAGTGAAAGCTGG	(433)	R-CCGCATGTCATAGGGACACGTC	(251)
NR2D		mGluR.5	
F-CCACCAGCAAAATCATGGTGCTG	64	F-ATCTCCCGATGTCAAGTGGTT	58,5
R-TGCAGCATCGTAGATGAAGGCGTC	(326)	R-GGACCATACTTCATCATCATC	(516)
NR3A		mGluR.6	
F-TTGACCGCCAACATATCCGAGCTA	58	F-GAGAACAACCGCAGGAACAT	56
R-ACACGTTTGGTCTTGAAATGCTGC	(353)	R-GCATACACCGCATCAATCAC	(185)
NR3B		mGluR.7	
F-CCGAGGCGTACATCAAGAAGAG	60	F-GCTCATCCAGAAGGACACCTCCGA	57
R-TTGGAGGTGAGCGGCGAGTT	(253)	R-CTACCATGGCCTGGGCTTGGAA	(257)
		mGluR.8	
		F-GCCCTTCTGACCAAAACAAA	60
		R-GTCCGCTGCTCTCCATAGTC	(197)

EAATs and GS	Annealing temperature (˚C) (Amplicon size, bp)	EAATs and GS	Annealing temperature (˚C) (Amplicon size, bp)
EAAT1		EAAT4	
F-TGGAGACTCTTACCCGAATCACAG	62	F-CAGCTGTGTACTACATGGTGAC	62
R-TGGCAAGACGATGACTGCGTG	(341)	R-CCGAGTGACATTTTCCAGGAAGC	(335)
EAAT2		EAAT5	
F- GGCAACTGGGGATGTACA	58	F-CTGCTGGAGAACAACCACATCGAC	62
R- ACGCTGGGGAGTTTATTCAAGAAT	(836)	R-CCAGCACGTTAATCATGGTGCGGA	(331)
EAAT3		GS	
F-TTCAGATGGCATAAACGTCCTG	60	F-GACCTTGTGAAGGAATCAGCATGG	62
R-GGAAAGGGTTCTTTCGTACGACTA	(326)	R-CCCTTTGAGTTACAATCGGGACAA	(598)

F, forward; R, reverse.

The possible importance of the Glu system in prostate cancer cells can be foreseen based on its relationship to prostate-specific membrane antigen (PSMA) expression. The cytoplasmic tail of PSMA associates with filamin A, a dimeric actin cross-linking protein stabilizing PSMA on the membrane (23). Moreover, a conserved tyrosine within the *C*-termini of mGluRs was identified to mediate binding to filamin-A (24), making it possible that a PSMA/mGluR complex could exist on the cells that express both molecules. PSMA, a type II trans-membrane protein consists of: i) a short intracellular amino terminal of 19 amino acids; ii) a transmembrane region of 24 amino acids; and iii) a glycosylated extracellular domain of 707 amino acids containing enzymatic activity as carboxypeptidase (23). PSMA possess both *N*-acetylated-γ-linked acidic dipeptidase (NAALADase) and folate hydrolase (FOLH) activities (24, 25). These two related peptidase activities hydrolyze γ-peptide bonds between *N*-acetyl-aspartate and glutamate in the abundant neuropeptide *N*-acetyl-aspartyl-glutamate (NAAG) and the γ-glutamyl linkages in pteroylpolyglutamate, respectively. Thus PSMA has been referred to alternatively as glutamate carboxypeptidase II (GCP-II) and folate hydrolase 1 (FOLH1). PSMA is overexpressed at different stages of prostate cancer, including the androgen ablation-independent disease stage, while its expression is increased on disease progression (26-29). Thus one of the constant products of PSMA enzymatic activity is glutamate, which might also be a potential modulator and functions through GluRs present in prostate cancer cells,

Table II. *Characteristics of PCR products produced by our specific PCR primers.*

Genes	Restriction enzyme	Restriction enzyme information	Undigested (amplicon size in bp)	Digested+ (amplicon fragments in bp)
NR1	Hae III	TAKARA (1051A)	280	172, 108
NR2A	MwoI	NEB (RO573S)	232	179, 53
NR2C	Hae III	TAKARA (1051A)	433	120, 120, 79, 66, 25, 23
NR2D	Hae III	TAKARA (1051A)	326	183, 68, 45, 30
NR3A	HINF I	TAKARA (1061A)	353	239, 114
NR3B	MWO I	NEB (RO573S)	253	185, 68*
MGLUR 1	HINF I	TAKARA (1061A)	237	178, 59
MGLUR 2	HINF I	TAKARA (1061A)	300	205, 95
MGLUR 3	HAE III	TAKARA (1051A)	220	134, 86
MGLUR 4	HAE III	TAKARA (1051A)	251	171, 80
MGLUR 5	MNL1	FERMENTAS (ER1071)	516	272, 244
MGLUR 6	HAE III	TAKARA (1051A)	185	125, 60
MGLUR 7	MWO I	NEB (RO573S)	257	135, 87, 39*
MGLUR 8	MNL1	FERMENTAS (ER1071)	197	121, 76
GS	HINF I	TAKARA (1061A)	598	312, 273, 13
EAAT1	HAE III	TAKARA (1051A)	343	208, 135
EAAT2	HIND III	TAKARA (1060A)	836	709, 127
EAAT3	HAE III	TAKARA (1051A)	326	231, 84, 11
EAAT4	HAE III	TAKARA (1051A)	335	149, 109, 77
EAAT5	MWO I	NEB (RO573S)	331	153, 78, 65, 35*

+Digestions carried out at 31°C except for those marked with* which took place at 60°C.

Table III. *Glu System expression in PC-3 and LNCaP cells.*

iGluRS	NR1	NR2A	NR2B	NR2C	NR2D	NR3A	NR3B
LNCaP (–DHT)	+	+	–	+	+	+	+
LNCaP (+DHT)	+	+	–	+	+	+	+
PC-3	+	+	–	+	+	–	+

mGluRs	mGluR.1	mGluR.2	mGluR.3	mGluR.4	mGluR.5	mGluR.6	mGluR.7	mGluR.8
LNCaP (–DHT)	+	+	+	+	+	+	–	+
LNCaP (+DHT)	+	+	+	+	+	+	+	–
PC-3	+	+	+	+	+	–	–	–

EAATs and GS	EAAT1	EAAT2	EAAT3	EAAT4	EAAT5	GS
LNCaP (–DHT)	+	+	+	–	+	+
LNCaP (+DHT)	+	+	+	+	+	+
PC-3	+	+	+	+	+	+

–, Not expressed; +, expressed; –DHT, no dihydrotestosterone; +DHT, dihydrotestosterone added.

signaling *via* PSMA-mediated mechanism as reported previously by a preliminary study (30). Alternatively, folates, which are produced as a by-product of this enzymatic reaction, could also be potential modulators for PSMA enzymatic activity. Therefore, the role of folate and glutamate on the invasiveness of PSMA-expressing cells has been targeted for further investigation (31).

Indeed, substrates that would inhibit PSMA activity have had an effect on the invasiveness of PSMA-expressing prostate cancer cells. Interestingly, small peptide substrates such as polygammaglutamated folates and *N*-acetylaspara-tylglutamate (NAAG) inhibitors have been identified, and their interaction site on the PSMA has been examined by X-ray crystallography (31, 32). Expression of PSMA in transfected PC-3 cells (PC-3 cells do not express PSMA) did reduce the invasion potential of PC-3 cells, suggesting that this reduction in the invasion capability was due to PSMA expression and not to intrinsic properties of transfected prostate cancer cell lines. Furthermore,

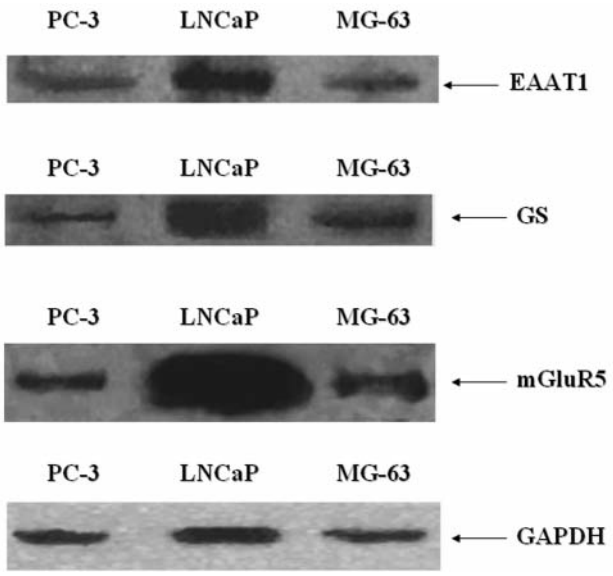

Figure 1. *An example of Western blot analysis in PC-3 and LNCaP prostate cancer cells assessing the expression of excitatory amino acid transporter type 1 (EAAT1), glutamine synthetase (GS) and metabotropic glutamate receptor 5 (mGluR.5). Note that all were expressed in both cell lines. However, LNCaP cells expressed higher levels of protein of these Glu system components. MG-63 cells were used as positive controls (22).*

knockdown of PSMA expression increased the invasiveness of LNCaP cells by 5-fold. Finally, expression of PSMA mutants lacking carboxypeptidase activity reduced the impact of PSMA expression on invasiveness. Thus, it seems that the PSMA enzymatic activity is associated with prostate cancer cell invasiveness while it is related to and/or regulated by glutamate concentrations (31).

The fact that mGluRs are expressed in human prostate cancer cells provides further insights for the role of G protein-coupled receptors (GPCRs) in prostate cancer cells. This hypothesis is corroborated by other reports which had suggested that GPCRs are involved in the neoplastic transformation of human prostate cancer (33). These data have documented: (i) elevated levels of certain enzymes, which in their turn can control the expression of various GPCRs in prostate cancer cells (34); (ii) increased production of GPCR ligands, such as endothelin 1 (ET-1) in prostate cancer cells and tissues (35, 36); and (iii) increased expression of orphan prostate-specific GPCRs, such as PSMA, in prostate cancer tissues compared to that in benign prostatic hyperplasia (37). In the classical paradigm, GPCR-mediated signal transduction implicates the agonist-dependent interaction of GPCRs with G proteins at the plasma membrane, and the subsequent generation, by membrane localised effectors, of soluble second messengers or ion currents. Rapid termination of GPCR signaling is produced by the phosphorylation of agonist-occupied receptors by members of the GPCR kinase (GRK) family and subsequent binding of β-arrestin proteins (38, 39). Binding of β-arrestin proteins to receptor uncouples the receptor from its cognate G protein, resulting in an attenuation of responsiveness of the signaling pathway of the agonist. The β-arrestin initiates the process of 'desensitization' by targeting it to clathrin-coated pits for internalization (40). Sequestered receptors are either dephosphorylated and recycled to the cell surface, or targeted for degradation.

In vitro studies have already documented that GPCR ligands, such as acetylcholine (41), angiotensin (42, 43), bombesin (42, 44-47), bradykinin (48-50), ET-1 (35, 51), FSH (36), isoproterenol (52), lysophosphatidic acid (LPA) (53-55), prostaglandins (56) and throbin (57), stimulate the proliferation of prostate cancer cells *via* intracellular signaling that involves mainly phosphorylation of extracellular signal-regulated kinases (ERKs) (58, 59). Notably mGluRs, such as mGluR5, have shown continuous or constitutive activity (*i.e.* are active in the absence of ligand or are activated continuously by abundant ligand production), a property which is intracellularly regulated by a cross-talking signal (59). Therefore, glutamate-mediated activation of GPCRs represents an alternative bioregulation system possibly affecting cancer cell biology. However, it is important to appreciate that the prostate cancer cell membrane is armed with many GPCRs, which are simultaneously activated by various extracellular stimuli and collectively control cell response. In concert with this, exposure of PC-3 cells to lysophosphatidic acid (LPA) elicited a significant increase of epidermal growth factor receptor (EGFR) sensitivity to exogenous EGF *via* transactivation of EGFR (55). Similarly, EGFR has been shown to cross talk to androgen receptor (AR) signaling pathways, as other growth factor receptors do [*e.g.* the insulin-like growth factor 1 receptor (IGF-1R)], activating intracellular pathways that involve G protein signaling too (33, 60-64).

In conclusion, the expression of components of the Glu system in prostate cancer cells is an important finding which may implicate the Glu system as an important and alternative bioregulatory mechanism for prostate cancer cell biology. To understand the potential regulatory role of the Glu system in cancer biology, extensive research should be directed both at the specific molecular-signaling pathways that are activated by GluRs in prostate cancer cells as well as on the cross-talk of mGluRs with other signaling pathways which have particular importance for prostate cancer growth and apoptosis. Conceivably, the Glu signaling pathways may become targets for the development of new anticancer therapies in prostate cancer.

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