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Research paper

Extracellular cysteine (Cys)/cystine (CySS) redox regulates metabotropic glutamate receptor 5 activity

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ABSTRACT

Extracellular cysteine (Cys)/cystine (CySS) redox potential (E_h) has been shown to regulate diverse biological processes, including enzyme catalysis, gene expression, and signaling pathways for cell proliferation and apoptosis, and is sensitive to aging, smoking, and other host factors. However, the effects of extracellular Cys/CySS redox on the nervous system remain unknown. In this study, we explored the role of extracellular Cys/CySS E_h in metabotropic glutamate receptor 5 (mGlu5) activation to understand the mechanism of its regulation of nerve cell growth and activation. We showed that the oxidized Cys/CySS redox state (0 mV) in C6 glial cells induced a significant increase in mGlu5-mediated phosphorylation of extracellular signal-regulated kinase (ERK), blocked by an inhibitor of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (MEK), U0126, a nonpermeant alkylating agent, 4-acetamide-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS), and a specific mGlu5 antagonist, 2-methyl-6-(phenylethynyl)pyridine (MPEP), respectively. ERK phosphorylation under oxidized extracellular Cys/CySS E_h was confirmed in mGlu5-overexpressed human embryonic kidney 293 (HEK293) cells. Oxidized extracellular Cys/CySS E_h also stimulated the generation of intracellular reactive oxygen species (ROS) involved in the phosphorylation of ERK by mGlu5. Moreover, activation of mGlu5 by oxidized extracellular Cys/CySS E_h was found to affect expression of NF-κB and inducible nitric oxide synthase (iNOS). The results also showed that extracellular Cys/CySS E_h involved in the activation of mGlu5 controlled cell death and cell activation in neurotoxicity. In addition, plasma Cys/CySS E_h was found to be associated with the process of Parkinson's disease (PD) in a rotenone-induced rat model of PD together with dietary deficiency and supplementation of sulfur amino acid (SAA). The effects of extracellular Cys/CySS E_h on SAA dietary deficiency in the rotenone-induced rat model of PD was almost blocked by MPEP pretreatment, further indicating that oxidized extracellular Cys/CySS E_h plays a role in mGlu5 activity. Taken together, the results indicate that mGlu5 can be activated by extracellular Cys/CySS redox in nerve cells, which possibly contributes to the process of PD. These *in vitro* and *in vivo* findings may aid in the development of potential new nutritional strategies that could assist in slowing the degeneration of PD.

Abbreviations: AMS, 4-Acetamide-4′-maleimidylstilbene-2,2′-disulfonic acid, disodium salt; BCA, Bradford Protein Assay; CRD, cysteine-rich domain; Cys, cysteine; CySS, cystine; DAergic, dopaminergic; DCF, dichlorofluorescein; DMEM, Dulbecco's Modified Eagle's Medium; GNDF, glial derived neurotrophic factor; GPCR, G-protein-coupled receptor; GSH, glutathione; DMSO, dimethylsulfoxide; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HD, heptahelical transmembrane domain; HEK293, human embryonic kidney; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; iNOS, nitric oxide synthase; MANF, astrocyte-derived neurotrophic factor; MFB, medial forebrain bundle; mGlu5, metabotropic glutamate receptor 5; MPEP, 2-methyl-6-(phenylethyl)-pyridine; NAC, N-acetylcysteine; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; PD, Parkinson's disease; RFU, relative fluorescence units; ROS, reactive oxygen species; SAA, sulfur amino acid; SD, Sprague-Dawley; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, standard error of the mean; SNc, substantia nigra pars compacta; STS, staurosporine; TBST, tris-buffered saline with Tween; TH, tyrosine hydroxylase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

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1. Introduction

Reversible redox reactions of thiol/disulfide couples regulate diverse biologic processes, including enzyme catalysis, gene expression, and signaling for cell proliferation and apoptosis [1–3]. The cysteine (Cys)/cystine (CySS) redox couple represents the predominant low-molecular-weight thiol/disulfide pool found in plasma and is sensitive to aging, smoking, and other host factors [4,5]. These findings are intriguing particularly when considered in conjunction with data showing that alteration in extracellular Cys/CySS redox potential (E_h) can drive signal transduction [6]. Moreover, extracellular Cys/CySS redox state also has been implicated in cell growth and apoptosis [3,6,7]. Depressed thiols levels in plasma and tissues have been implicated in a number of human diseases such as Alzheimer's and Parkinson's, diabetes, cystic fibrosis, and HIV infection [8–11].

Metabotropic glutamate receptors (mGluRs), a type of G-protein-coupled receptor (GPCR), are a large family of surface receptors composed of multiple domains. They possess an extracellular Venus flytrap domain (VFT) where agonists bind and a heptahelical transmembrane domain (HD) is common to all GPCRs that is responsible for G-protein activation. For most of these receptors, a cysteine-rich domain (CRD) is linked to the two domains [12,13]. CRD is composed of nine highly conserved Cys and is known to be important for signal transduction in mGlu-like receptors [14]. mGlu5, a type of group I mGluR, is a disulfide-linked dimer [15], making it a good candidate for evaluating sensitivity to extracellular thiol/disulfide redox.

mGlu5, widely expressed in astrocytes, has been utilized as a target for pharmacotherapy in Parkinson's disease (PD) [16,17]. PD is a complex chronic neurodegenerative disorder primarily involving loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNc), decay of the nigrostriatal tract, symptomatic rigidity, bradykinesia, and resting tremor. As tyrosine hydroxylase (TH) catalyzes the formation of L-3,4-dihydroxyphenylalanine (L-DOPA), the rate-limiting step in the biosynthesis of dopamine, the disease can be considered a TH-deficiency syndrome. Moreover, loss of these neurons is also associated with non-neuronal pathology such as activated microglial cells and reactive astrocytes [18]. Glial cells may be a source of trophic factors and can protect against reactive oxygen species (ROS) and glutamate. They can also mediate a variety of deleterious events related to the production of reactive oxygen and nitrogen species of cytokines that may contribute to neuronal injury and cell death in PD [19].

Based on the above evidence, we hypothesized that extracellular Cys/CySS E_h could affect the activation of mGlu5 and may have a role in the pathogenesis of PD. We first tested this hypothesis by modifying extracellular Cys/CySS E_h and detecting the activation of mGlu5 in C6 glial cells. We found that alteration of extracellular Cys/CySS E_h was sufficient to alter phosphorylation of ERK, with the greatest phosphorylation observed under the oxidized condition (0 mV) through mGlu5 activation. Oxidized extracellular Cys/CySS redox state also promoted the production of ROS, the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and controlled the rotenone-induced cell death and activated the cells via induction of inducible nitric oxide synthase (iNOS). Next, the effects of plasma Cys/CySS E_h in a rotenone-induced rat model of PD were also examined. Our results may provide a novel mechanism by which mGlu5 can be activated by extracellular Cys/CySS redox state and a theoretical basis for understanding the regulation of mGlu5 activity in nerve cells and the pathogenesis of PD.

2. Materials and methods

2.1. Preparation of media and measurement of Cys/CySS E_h

Various extracellular thiol/disulfide redox potentials were established by varying the concentrations of Cys and CySS added to Cys-free Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 10 U/ml penicillin, and 10 μg/ml streptomycin (catalog no. 21012; Invitrogen, CA, USA), as previously reported [20]. Stock solutions for Cys and CySS (10 mM; pH 7.4) were made fresh before each experiment and filtered through a 0.2-μm syringe filter. To generate the desired redox state (0, −80, −150 mV), the amount of Cys/CySS added to the redox potential state was calculated using the Nernst equation, $E_h = -250 + 30\log[(\mathrm{CySS})/(\mathrm{Cys})^2]$ for pH 7.4.

2.2. Cell culture and treatment

C6 glial cells and human embryonic kidney 293 (HEK293) cells were maintained in complete medium (DMEM plus 10% fetal bovine serum and 1% penicillin/streptomycin) (Hyclone, USA) in a 37 °C/5% CO_2 incubator. To express the receptors in HEK293 cells, mGlu5 plasmid was mixed with Lipofectamine 2000 (Invitrogen) and added to serum-free medium on a tissue culture plate. Following 4 h incubation, fetal bovine serum was added to the medium for a final concentration of 10%. Cells were harvested after 48 h MN9D cells, a mouse dopaminergic neuronal cell line, were cultivated in complete medium (DMEM/F12 plus 10% fetal bovine serum and 1% penicillin/streptomycin) (1:1; Gibco, NY, USA). For neurotoxicity, C6 cells were incubated in various media of redox states ranging from 0 mV to −150 mV at 37 °C and 5% CO_2 for 6 h. Conditioned media from the C6 cells were added to the MN9D cells, which were incubated at 37 °C and 5% CO_2 for a further 6 h. For cell preparation, cells had been treated with the following agents: To block the ERK pathway, the cell culture was preincubated with U0126 (Tocris Biosciences, UK) 10 μM for 30 min. To inhibit receptors, the cell culture was treated with MPEP (Tocris Biosciences) 0.5 mM for 30 min before stimulation. Rotenone was prepared as 50 μM stock solutions, dissolved in dimethylsulfoxide (DMSO) and stored at −80 °C until use. For treatments, rotenone stock solution was diluted with medium to 50 nM, and added to the cells.

2.3. Western blot

After appropriate treatments, cells were lysed in lysis buffer [final concentration 50 mM Tris (pH 8.0), 50 mM NaCl, 1% Nonidet P-40, 20 nM okadaic acid, 20 μM sodium orthovanadate, phosphatase inhibitor mixture, and protease inhibitor mixture (Pierce, USA)]. The protein concentrations were determined by bicinchoninic acid (BCA) assay (Pierce). Immunoblot analysis was performed essentially as described previously [21], using equal amounts of protein for each sample loaded onto 8 or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Next, proteins were transferred onto immobilon-P transfer membranes (Millipore, Bedford, USA). The membranes were then blocked for 1 h with TBST (20 mM Tris–Cl, pH 7.6, 137 mM NaCl, 0.05% Tween 20) and 5% milk, and incubated with the indicated antibodies overnight. The blots were then rinsed again with TBST and incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology, USA) for 2 h at room temperature. The blots were then rinsed with TBST several times and developed using a luminescent chromogen SuperSignal West Femto (Pierce, USA). The following antibodies were used for the Western blot analysis: