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Perspective

Metabotropic glutamate receptor 5 mediates the suppressive effect of 6-OHDA-induced model of Parkinson's disease on liver cancer

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ABSTRACT

Numerous epidemiological studies suggested that there is a variable cancer risk in patients with Parkinson's disease (PD). However, the underlying mechanisms remain unclear. In the present study, the role of metabotropic glutamate receptor 5 (mGluR5) has been investigated in 6-hydroxydopamine (6-OHDA)-induced PD combined with liver cancer both *in vitro* and *in vivo*. We found that PD cellular model from 6-OHDA-lesioned MN9D cells suppressed the growth, migration, and invasion of Hepa1-6 cells *via* down-regulation of mGluR5-mediated ERK and Akt pathway. The application of 2-methyl-6-(phenylethyl)-pyridine and knockdown of mGluR5 further decreased the effect on Hepa-1-6 cells when co-cultured with conditioned media. The effect was increased by (*S*)-3,5-dihydroxyphenylglycine and overexpression of mGluR5. Moreover, more release of glutamate from 6-OHDA-lesioned MN9D cells suppressed mGluR5-mediated effect of Hepa1-6 cells. Application of riluzole eliminated the increased glutamate release induced by 6-OHDA in MN9D cells and aggravated the suppressive effect on Hepa-1-6 cells. In addition, the growth of implanted liver cancer was inhibited in 6-OHDA induced PD-like rats, and was associated with increased glutamate release in the serum and down-regulation of mGluR5 in tumor tissue. Collectively, these results indicate that selective antagonism of glutamate and mGluR5 has a potentially beneficial effect in both liver cancer and PD, and thus may provide more understanding for the clinical investigation and further an additional therapeutic target for these two diseases.

1. Introduction

Previous studies have provided evidence for an influence by Parkinson's disease (PD) on cancer risk, with the overall incidence of cancer reduced in PD patients, including smoking-related cancers such as lung, larynx and urinary bladder as well as nonsmoking-related cancers such as prostate and colon, and with the increased incidence of malignant melanoma, non-melanocytic skin cancer and breast cancer [1–4]. Interestingly, Wirdefeldt et al. revealed a modestly increased cancer risk among patients up to one year before or after the index date for PD, but an overall lower risk from one year after the index date onward [5]. Thus, the relationship between these two diseases and the potential mechanisms involved in this association requires further investigation.

Neuronal degeneration is known to result from excessive excitotoxicity caused by increased glutamate synthesis [6], with regulation of glutamate receptor activity reported to relieve symptoms of PD using dopamine-replacement drugs [7,8]. Metabotropic glutamate receptors (mGluRs) belong to the G-protein coupled receptors family. Eight different types of mGluRs exist that can be classified into three groups based on receptor structure and physiological activity. Group I mGluRs, mGluR1 and mGluR5 have been implicated in a variety of neurological disorders, including PD, amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) [9]. In experimental models of PD, blockade of mGluR5 by application of 2-methyl-6- (phenylethynyl) pyridine (MPEP) has been shown to reduce the loss of striatal tyrosine hydroxylase (TH)-

Abbreviations: PD, Parkinson's disease; 6-OHDA, 6-hydroxydopamine; mGluR5, metabotropic glutamate receptor 5; TH, tyrosine hydroxylase; MPEP, 2-methyl-6-(phenylethyl)-pyridine; DHPG, (*S*)-3,5-dihydroxyphenylglycine; CREB, cAMP-response element binding protein; ERK, extracellular signal-regulated kinase; Ki-67, nuclear-associated antigen Ki-67; MMP, Matrix metalloproteinase; PCNA, proliferating cell nuclear antigen; Bcl-2, B-cell lymphoma-2.

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immunopositive fibers and nigral dopamine (DA) neurons [10]. Accordingly, glutamate receptors have been used as a therapeutic target for PD [11,12].

It has been reported that mGluR5 plays an important role both in central nervous system (CNS) and peripheral cancers [13–15]. Autocrine and paracrine glutamate accumulated in glioma and adjacent non-tumoral tissue has been reported to promote the proliferation and invasion of this malignant tumor [16,17]. In keeping with this, expression of mGluR5 has been observed in oral squamous cell carcinoma, with inhibition of mGluR5 found to decrease the proliferation of malignant cells [14]. Recent study has shown that selective blockade of mGluR5 was found to protect liver damage against increases in aspartate and alanine aminotransferase activity in mouse models of fulminant hepatic failure and the formation of liver fibrosis [18]. Additionally, inhibition of mGluR5 was found to impair the production of inducible nitrogen oxide synthase and reactive oxygen species in a mouse model of acetaminophen-induced hepatotoxicity [19], and to protect rat hepatocytes from hypoxic damage [20]. Previous study in our laboratory has shown that inactivation of mGluR5 by MPEP causes suppressive effect on hepatocellular carcinoma *in vitro* and *in vivo* [21]. These studies suggest that mGluR5 activity may contribute to the development of liver cancer. Therefore, we hypothesized that PD may influence the development of liver cancer by regulating the activity of mGluR5.

The aim of this study was to investigate the role of mGluR5 in the effects of PD on growth, migration, and invasion of liver cancer *in vitro* and *in vivo*. Our study reveals a novel role for mGluR5 in the association between PD and liver cancer, and suggests that mGluR5 may be a crossover molecule in pathology of these two conditions. As such, mGluR5 may provide a potential novel therapeutic target in both PD and liver cancer.

2. Materials and methods

2.1. Cell culture

The mouse hepatoma cell line (Hepa1-6) and the rat LLC-WRC256 carcinosarcoma cell line were purchased from American Type Culture Collection (ATCC, USA). The mouse dopaminergic neuronal cell line (MN9D) was a generous gift from Professor Hui Yang (Capital Medical University, Beijing, China). Hepa1-6 cells and MN9D cells were grown in Dulbecco's modified Eagle's medium/NutMixF-12 supplemented with GlutaMax-1 (DMEM/F-12 GlutaMax, devoid of glutamine and glutamate, Gibco, USA), containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS). LLC-WRC256 carcinosarcoma cells were grown in M199 complete growth medium (Gibco, USA) containing 5% horse serum and 1% PS. All cell lines were maintained in an incubator at 37 °C with 5% CO2. MN9D cells at >90% confluence were serum starved for 12 h prior to exposure to 6-OHDA (Sigma, USA) (25–200 μM) for 15 min, before being cultured in new serum-free DMEM/F-12 GlutaMax media for 12–24 h as previously described [22]. The supernatant was collected and centrifuged at 133 × g for 3 min, and prepared for use as conditioned media. Hepa1-6 cells were cultured with conditioned media for 12–48 h. To activate or antagonize mGluR5, Hepa1-6 cells were treated with 100 μM (S)-3, 5-dihydroxyphenylglycine (DHPG, Tocris Biosciences, USA) or 100 μM MPEP (Tocris Biosciences, USA), according to previously published protocols [21].

2.2. Knockdown and overexpression of mGluR5

Short hairpin RNA (shRNA) duplexe directed against mGluR5 was as follow: TGCTGTTGACAGTGAGCGAACGCAGAGTTCTTCATCGTTATAGTGAAGCCACAGATGTATAACGATGAAGAACTCTGCGTGTGCCTACTGCCTCGGA. Scramble shRNA (TGCTGTTGACAGTGAGCGCCCGGCTGAAGAGCCTGATCAATAGTGAAGCCACAGATGTATTGATCAGGCTCTTCAGCCGGTTGCCTACTGCCTCGGA) was used as a control in transfection experiments. Both of the shRNAs were synthesized by Beifang Yitao Co., Ltd (Beijing, China). The cDNAs of rat Flag-mGluR5 was kindly provided by Prof. Junqi He (Capital Medical University, Beijing, China).

To knockdown or overexpress mGluR5 in Hepa1-6 cells, sh-mGluR5 or Flag-mGluR5 plasmid was mixed with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) and added to 2 mL of serum-free medium in 35 mm dishes. After 4 h incubation, fetal bovine serum was added to the medium at a final concentration of 10%. Twenty-four hours later, the transfection efficiency of cells was detected.

2.3. MTS assay

Cell proliferation activity was evaluated using 3-(4,5-dimethylthiazol-2-yl)- 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Cell Tilter 96 Aqueous Assay, Promega, USA). Hepa1-6 cells were collected following trypsinization and plated in 96-well plates at a density of 5000 cells per well. Once cells reached 70%–80% confluence, the medium was replaced with conditioned media in the absence or presence of MPEP and DHPG. Prior to analysis, MTS solution was added to cells and incubated for 1 h at 37 °C and the absorbance was measured at 490 nm on a microplate reader (Elx800, Bio-Tek Instruments Inc, USA).

2.4. Wound-healing assay

Cell migration was analyzed by an *in vitro* wound-healing assay. Hepa1-6 cells were seeded into 6-well plates at a density of 5×10^5 cells per well, and a scratch wound in the cell monolayer was made using a pipette tip when cells reached 90% confluence. After washing away all detached cells with phosphate buffered Saline (PBS), the medium was replaced with conditioned media for 6–24 h in the presence or absence of MPEP and DHPG. The distances of wounds were measured by the microscope at 0, 6, 12, and 24 h following treatment. Cell migration was evaluated according to the following formula: Cell motility = $(\text{distance}_{\text{experimentaltimepoint}} - \text{distance}_{0h})/\text{distance}_{0h}$.

2.5. Western blot analyses

After appropriate treatments, cells and tissues were lysed and homogenized. The proteins were subjected to western blot analysis as reported [25]. Reactive signal bands were visualized using enhanced chemiluminescence kit (Millipore Corporation, USA), and image acquisition was done using Amersham imager 600 (GE, USA). Signal intensity was semi-quantified with Image J software (National Institutes of Health, Bethesda, MD, USA). The following primary antibodies were used according to the manufacturers' instructions: anti-mGluR5 (Abcam, UK), anti-β-actin, anti-GAPDH, anti-Bcl-2, anti-Bax, anti-PCNA, anti-MMP-9, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-CREB, anti-CREB, anti-phospho-mTOR, anti-mTOR, anti-phospho-GSK-3β, anti-GSK-3β, anti-phospho-Akt and anti-Akt (All from Cell Signaling Technology, USA).

2.6. Detection of glutamate concentration

For all *in vitro* experiments, the resultant co-cultured media were collected after Hepa1-6 cells were cultured with various