Group I mGluR ligands fail to affect 6-hydroxydopamine-induced death and glutamate release of PC12 cells[1]

LI Hao, DING Jian-Hua, HU Gang[2]

Department of Pharmacology & Neurobiology, Nanjing Medical University, Nanjing 210029, China

KEY WORDS metabotropic glutamate receptors; PC12 cells; cell survival; 6-hydroxydopamine; glutamic acid; excitatory amino acid agonist; excitatory amino acid antagonist

ABSTRACT

AIM: To study the effect of group I metabotropic glutamate receptor (mGluR) ligands on 6-hydroxydopamine (6-OHDA)-induced death and glutamate release of PC12 cells. **METHODS:** PC12 cells were exposed to 100 μmol/L of group I mGluR agonist (*RS*)-3,5-dihydroxy-phenylglycine (DHPG) or antagonist *DL*-2-amino-3-phosphonopropionic acid (*DL*-AP3) 1 h before addition of 6-OHDA 100 μmol/L. After incubation for 24 h, morphological alterations were observed with microscope, DNA fragmentation was detected by terminal deoxynucleotidyl transferse-mediated dUTP nick end-labeling (TUNEL) method, cytotoxicity was measured by MTT assay, and glutamate release was assayed by high performance liquid chromatography. **RESULTS:** 6-OHDA decreased cell viability ($P<0.01$) and induced concentration- and time-dependent glutamate release from PC12 cells. Group I mGluR ligands did not affect 6-OHDA-induced death of PC12 cells and had no influence on glutamate levels. **CONCLUSION:** Group I mGluR ligands cannot protect PC12 cells from 6-OHDA-induced death.

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder. The primary pathological change of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta. A role for excitotoxicity in the etiology or progression of PD has been proposed in recent years. Glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system. There are two classes of glutamate receptors: ionotropic glutamate receptors and metabotropic glutamate receptors (mGluR). mGluR could be subdivided into three groups: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8)[1]. Although the role of mGluR in neurological disorders has been widely investigated, their effect in PD has not to be determined. Recent studies showed that dopamine modulated the function of group II and group III metabotropic glutamate receptors in the substantia nigra pars reticulata[2]. In methamphetamine-sensitized rats, both metabotropic glutamate I and II receptors mediated augmentation of dopamine release from the striatum[3], suggesting that mGluR may take part in the pathogenesis of PD.

6-Hydroxydopamine (6-OHDA), a hydroxylated derivative of dopamine, is widely used for animal models of PD. *In vitro*, this neurotoxin triggers apoptosis of catecholaminergic PC12 cell line[4]. *In vivo*, 6-OHDA lesion induces apoptosis of dopaminergic cells in the

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[2] Correspondence to Prof HU Gang. Phn 86-25-666-3108. Fax 86-25-652-8460. E-mail ghu@njmu.edu.cn
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rat substantia nigra and common features of PD[5]. mGluR5 antagonist 2-methyl-6-phenylethynyl-pyridine (MPEP) can alter motor behaviour such as rotarod, locomotor activity, and rotational responses in unilateral 6-OHDA-lesioned rats[6]. But it is still unknown about the effect of group I mGluR ligands on 6-OHDA-induced death of dopaminergic cells.

PC12 cells express group I mGluRs[7]. The present study was aimed to clarify the effect of group I mGluR ligands on 6-OHDA-induced death and glutamate release of PC12 cells in an attempt to further characterize the role of mGluR in PD.

MATERIALS AND METHODS

Reagents Group I mGluR agonist (*RS)*-3,5-dihydroxyphenylglycine (DHPG) and antagonist *DL*-2-amino-3-phosphonopropionic acid (*DL*-AP3) were provided by Tocris (UK). 6-OHDA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA). *In situ* cell death detection kit (POD) was obtained from Roche (USA). *o*-Phthalaldehyde (OPA) was provided by Fluka (Switzerland). Dullbecco's modified Eagle's medium (DMEM) was obtained from Life Technology. PC12 cells were purchased from American Type Culture Collection.

Cell culture PC12 cells were routinely grown in DMEM consisting of benzylpenicillin 50 kU/L, 50 mg/L of streptomycin, and 10 % (v/v) heat-inactivated fetal bovine serum in a humidified atmosphere of 5 % CO_2 at 37 °C.

Drug treatments Cells were plated at the density of 1×10^5 cells/cm^2 on 24-well dishes. Twenty hours later, cells were treated with group I mGluR agonist DHPG 100 μmol/L or antagonist *DL*-AP3 1 h before addition of 6-OHDA 100 μmol/L. The plates were further incubated at 37 °C for 24 h .

Apoptosis assay To detect apoptotic cell death, cells were stained using an '*in situ* cell death detection kit, POD'. Briefly, cells were fixed for 1 h in 4 % paraformaldehyde in phosphate buffered saline at room temperature. Endogenous peroxidase was inactivated by incubation with 2 % hydrogen peroxide dissolved in methanol for 10 min at room temperature. Cells were permeabilized with 0.1 % Triton X-100 for 2 min on ice. After that, cells were labeled by incubation with TUNEL reaction mixture at 37 °C for 1 h. The cells were washed three times with phosphate buffered saline and then incubated with peroxidase-converter at 37 °C for 30 min. Then cells were washed and stained with fresh 0.05 % diaminobenzidine (DAB).

Cytotoxicity assay Cells were placed at the density of 1×10^5 cells/cm^2 in 96-well plates. At the end of drug treatments, media were discarded, and 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (dissolved at 5 g/L in PBS) was added to each well. After incubation at 37 °C for 4 h, the supernatants were discarded and 200 μL of acidified isopropanol was added and mixed to thoroughly dissolved resultant dark blue crystal. The absorbance (*A*) in each well was determined with an automatic plate reader (Clinibio128C, Austria) with a 570-nm test wavelength.

Quantification of glutamate At stated time points, media were withdrawn and $HClO_4$ 1 mol/L was added at a ratio of 3:2 (v/v), centrifuged (5000×*g*, 4 °C) for 5 min. And then $KHCO_3$ 2 mol/L was added to the supernatants at a ratio of 3: 4 (v/v), centrifuged again and the supernatants were collected for glutamate assay. Derivatization stock solution was prepared by dissolving OPA 25 mg in 1 mL methanol, after which 5 μL betamercaptoethanol and 9 mL sodium teraborate 0.1 mol/L (pH 9.3) were added. Before usage, stock solution was diluted with sodium teraborate 0.1 mol/L (pH 9.3) at a ratio of 1:3 (v/v). Samples were mixed with equal volume of derivatization solution, and reacted at 32 °C for 4 min. The mobile phase [methanol 25 % (v/v), Na_2HPO_4 0.1 mol/L, pH 6.75] was delivered to the analytical column (C18, 150 mm×4.6 mm) at a rate of 1.0 mL/min. The electrochemical detector (BAS LC-4C, USA) was set at 0.7 V.

Data analysis All values were presented as mean±SD. Student's two-tailed *t*-test was used for comparison and the difference was considered significant if $P<0.05$.

RESULTS

Effect of DHPG or *DL*-AP3 on 6-OHDA-induced morphological alterations of PC12 cells Under phase contrast microscope, untreated PC12 cells were in long fusiform shape with slender processes. After exposure to 6-OHDA 100 μmol/L for 24 h, most cells shrunk and some even fell into the medium. Pretreatment with DHPG or *DL*-AP3 100 μmol/L had no effect on 6-OHDA-induced morphological alterations (Fig 1).

6-OHDA induced apoptosis of PC12 cells Chro-

matin condensation and DNA fragmetation could be seen after exposure to 6-OHDA 100 μmol/L for 24 h (Fig 2).

Effect of DHPG or *DL*-AP3 on cell viability Treatment with DHPG or *DL*-AP3 100 μmol/L alone did not alter cell viability. Exposure to 6-OHDA 100 μmol/L for 24 h induced a strong diminution of cell viability ($P<0.01$). Pretreatment with DHPG or *DL*-AP3 100 μmol/L neither alleviated nor aggravated the cytotoxicity of 6-OHDA (Fig 3).

Effect of DHPG or *DL*-AP3 on 6-OHDA-induced glutamate release from PC12 cells 6-OHDA induced glutamate release from PC12 cells in a concentration- and time-dependent manner (Tab 1, 2). Treatment with DHPG or *DL*-AP3 100 μmol/L alone did not increase glutamate release compared with control. Exposure to 6-OHDA 100 μmol/L for 24 h strongly enhanced glutamate release ($P<0.01$). Pretreatment with DHPG or *DL*-AP3 100 μmol/L neither increased nor decreased 6-OHDA-induced glutamate release from PC12 cells (Fig 4).

Tab 1. Concentration-dependent glutamate release induced by 6-OHDA from PC12 cells. *n*=6. Mean±SD. $^cP<0.01$ *vs* control.

Treatment	Glu concentration/μmol·L^{-1}
Control	20.3±1.3
6-OHDA	
50 μmol/L	56±3^c
100 μmol/L	86±6^c
150 μmol/L	99±12^c

Tab 2. Time-dependent glutamate release induced by 6-OHDA 100 μmol/L. *n*=6. Mean±SD. $^cP<0.01$ *vs* control.

Time	Control/μmol·L^{-1}	6-OHDA/μmol·L^{-1}
45 min	11.2±2.8	13.0±2.3
1.5 h	11±3	20.4±2.0^c
3 h	16±4	32±5^c
6 h	22±5	56±6^c
18 h	21±4	73±4^c
24 h	20.3±1.3	86± 6^c

DISCUSSION

Several studies suggest a role for group I mGluR in regulation of neuronal cell death. Group I mGluR agonists protected cultured cerebellar granule cells from oxygen-glucose deprivation-induced death[8]. Endogenous activation of mGluR1a contributed to the development of neuronal degeneration of excitotoxic origin, and the selective mGluR1a antagonist had a neuroprotective activity[9]. However, very little is known about the effect of these receptors in the case of PD. Immunocytochemistry and unbiased stereology demonstrated that group I mGluR antagonist AIDA protected nigral dopamine cells from MPTP-induced injury[10]. The present study showed that neither group I mGluR agonist nor antagonist had an effect on 6-OHDA-induced death of PC12 cells. Because the oxidative stress induced by 6-OHDA was so strong, it prohibited the effect of group I mGluR. On the other hand, since DHPG and *DL*-AP3 can act on both mGluR1 and mGluR5, the effect of each subtype can not be distinguished. Moreover it is possible that the amount of mGluR expressed in PC12 cells was not enough to show any detectable effect.

Our data showed that 6-OHDA induced glutamate release from PC12 cells in a concentration- and time-dependent manner. Since glutamate is the main excitatory amino acid in the central nervous system, it can be speculated that glutamate is involved in the cytoxicity mechanism of 6-OHDA. Release of glutamate, dopamine, serotonin, acetylcholine, and some other neurotransmitters could be regulated by several mGluR ligands[1]. But in the present study, DHPG or *DL*-AP3 neither increased nor decreased 6-OHDA-induced glutamate release from PC12 cells. This may be due to the altered activity of calcium or potassium channels by the large numbers of reactive oxygen species produced by 6-OHDA, which made the channels lose responsibility to these ligands.

Our data suggested that group I mGluR ligands could not protect PC12 cells from 6-OHDA-induced death. *In vivo* studies based on this experiment will gain more insight into the relationship of mGluR and PD.

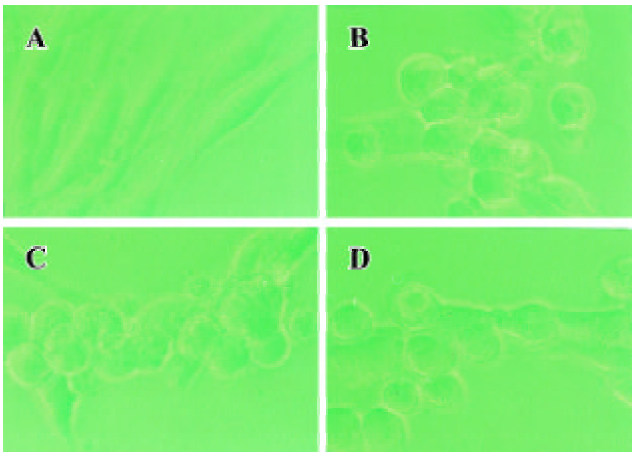

Fig 1. Effect of DHPG or *DL*-AP3 on 6-OHDA-induced morphological alterations in PC12 cells. Under phase contrast microscope PC12 cells were in long fusiform shape with slender processes (A). Treatment with 6-OHDA 100 μmol/L made most cells shrink (B). Pretreatment with DHPG or *DL*-AP3 100 μmol/L had no effect on 6-OHDA-induced morphological alterations (C and D). ×400.

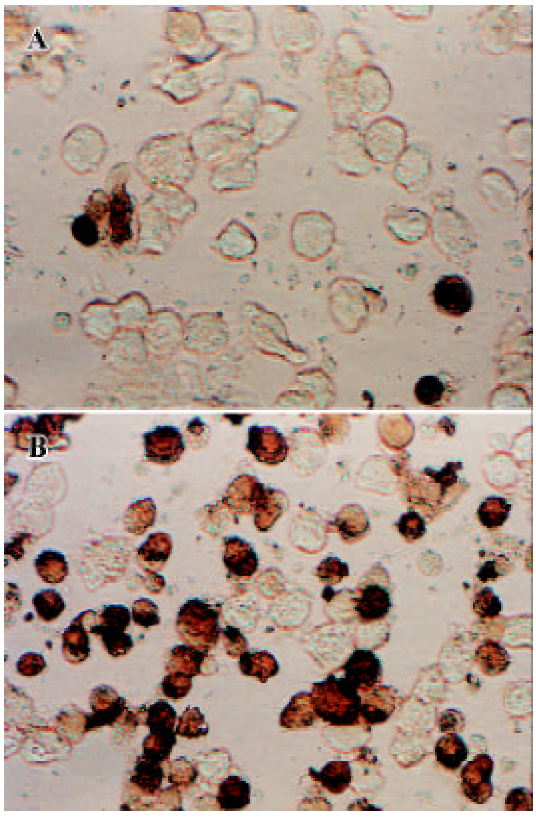

Fig 2. TUNEL staining of PC12 cells before (A) and after exposure to 6-OHDA 100 μmol/L for 24 h (B). DNA fragmentation could be seen after exposure to 6-OHDA. ×400.

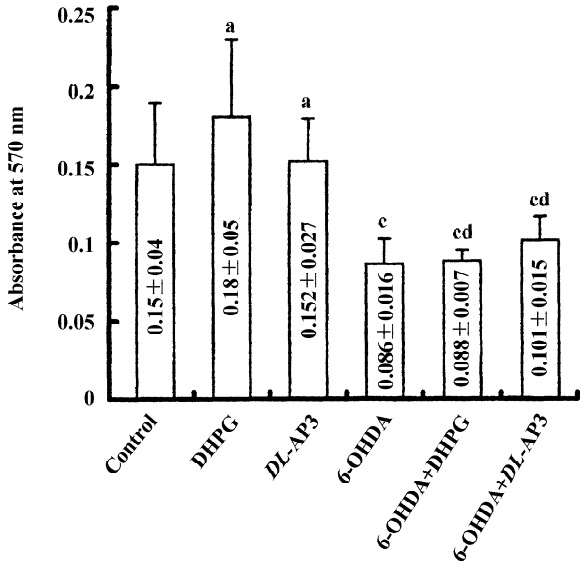

Fig 3. Cell viability assayed by MTT method. *n*=6. Mean±SD. [a]*P*>0.05, [c]*P*<0.01 *vs* control. [d]*P*>0.05 *vs* 6-OHDA.

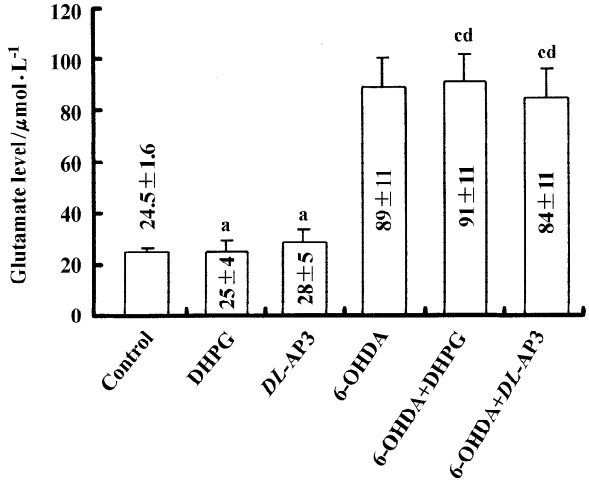

Fig 4. Effect of DHPG or *DL*-AP3 100 μmol/L on 6-OHDA-induced glutamate release from PC12 cells. *n*=6. Mean±SD. [a]*P*>0.05, [c]*P*<0.01 *vs* control. [d]*P*>0.05 *vs* 6-OHDA.

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