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L-Pyroglutamic acid protects rat cortical neurons against sodium glutamate-induced injury

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KEY WORDS pyrrolidonecarboxylic acid; sodium glutamate; calcium; nitric oxide

ABSTRACT

AIM: To evaluate the effects of *L*-pyroglutamic acid (*L*-PGA, *L*-5-oxo-2-pyrrolidinecaroxylic acid) on sodium glutamate-induced neurotoxicity in rat cortical neurons. **METHODS**: In primary cortical cultures from 16-d-old fetal rat, neuronal viability and contents of nitrite in the bathing medium after transient exposure to sodium glutamate (Glu) were measured; with Fura 2-AM as an intracellular calcium indicator, AR-CM-MIC cation measurement system was used to examine cytosolic free calcium ($[Ca^{2+}]_i$). **RESULTS**: *L*-PGA 10 - 80 $\mu mol \cdot L^{-1}$, inhibited Glu (500 $\mu mol \cdot L^{-1}$)-induced neuronal loss in a concentration-dependent manner with IC_{50} value of (41 ± 9) $\mu mol \cdot L^{-1}$ (95 % confidence limits: 30.3 - 54.7 $\mu mol \cdot L^{-1}$). *L*-PGA also attenuated Glu-induced NO release. *L*-PGA 1, 3, 10, 30, and 100 $\mu mol \cdot L^{-1}$ depressed Glu-caused $[Ca^{2+}]_i$ elevation by 20.5 %, 34.4 %, 47.7 %, 70.6 %, and 80.4 %, respectively. **CONCLUSION**: *L*-PGA protects cortical neurons against Glu-induced neurotoxity which may be related to inhibition of NO formation or suppression of the rise in $[Ca^{2+}]_i$.

INTRODUCTION

Large amounts of evidence implicate endogenous excitatory amino acids, especially glutamic acid, in a number of neurological diseases. Overactivation of glutamate receptors is involved in the pathogenesis of either acute neuronal injury such as stroke or chronic neurodegenerative disorders including Alzheimer's disease, Huntington's disease, and Parkinson's disease[1,2]. Excitotoxic damage is mediated in large part by uncontrolled Ca^{2+} influx resulting in sustained elevation of $[Ca^{2+}]_i$, leading to the activation of Ca^{2+}-dependent protease, kinase, and lipase, cell damage, and eventually cell dealth. Excessive production of NO has been shown to underlie at least in part glutamate-mediated neuronal toxicity in cultures of cortical neurons[3].

L-Pyroglutamic acid (*L*-PGA, *L*-5-oxo-2-pyrrolidinecaroxylic acid) is a cyclic derivative of glutamic acid and presents in a free form in the mammalian brain, plasma, and cerebrospinal fluids. *L*-PGA is one of the active components in several traditional Chinese medicines such as *Panax ginseng* and *Ophiopogon japonicus*[4,5]. *L*-PGA improved learning and memory capacity in aged rats[6], increased the blood flow and facilitated the uptake and utilization of glucose by brain tissue[7], exerted an anxiolytic activiy[8], antagonized the sodium glutamate (Glu)-induced seizures in mice[9]. It seems that *L*-PGA has the ability to block glutamate receptors[9,10]. Furthermore, *L*-PGA and its derivatives may also be used as potential drug carriers which facilitate diffusion of drug across the blood-brain-barrier and enhance the rate of entry into the central nervous system[11]. However, it has not been reported whether *L*-PGA has neuroprotective effects and the molecular mechanisms involved have been pharmacologically limited by the possible effects of systemic metabolism or blood flow that might complicate the studies *in vivo*.

The present study was to determine whether *L*-PGA could protect cortical neuron against Glu insults. The release of NO and neuronal intracellular calcium levels were measured to explore its mechanism of action.

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MATERIALS AND METHODS

Cell culture Cortical neuronal cultures were prepared from fetal Sprague-Dawley rats (16 d after gestation) neocortices[12]. After removal of meninges, the tissues pooled from 12 fetal rats were minced and digested by trypsin 0.25 g·L^{-1} at 37 ℃ for 20 min. The digestion was terminated by adding Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10 % heated-inactivated fetal bovine serum (FBS). The dispersed cells were seeded into 15 mm-multiwell plate (NUNC) at the density of 1.5 × 10^5 cells per well. Cultures were incubated at 37 ℃ containing 5 % CO_2. The culture medium was renewed twice a week. To prevent the proliferation of nonneuronal cells, cytosine arabinoside 10 μmol·L^{-1} was added d 7 for 48 h. Cultures were selected for study 14 – 16 d after seeding.

Experiments with Glu and *L*-PGA The attached cells were washed twice with PBS. After the cells were incubated with Lock's solution without Mg^{2+} (NaCl 154, KCl 5.6, $CaCl_2$ 2.3, $NaHCO_3$ 3.6, and HEPES 5.0 mmol·L^{-1}, pH 7.4) for 30 min, then Glu 500 μmol·L^{-1} was added. Thirty min later, the reaction was terminated by washing the monolayer cells twice with the Lock's solution and then cultures were kept at 37 ℃ for 24 h. For preincubation with *L*-PGA (donated by Kunming Institute of Botany, Chinese Academy of Sciences), the medium was removed and after washing, different concentrations of *L*-PGA were added and incubated at 37 ℃ for 20 min before Glu was added.

Trypanblau staining, MTT colorimetric assay and NO efflux assay The dead cells were stained by 1.5 % trypanblau, and viability rate was the percentage of unstained cells to total cells. Active mitochondria of living cells can cleave 3-(4, 5-dimethylthiazoyl-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Fluka) and produce formazam, the amount of which is directly proportional to the cell number. MTT was added to all wells (final concentration 0.5 g·L^{-1}) and the cultures were incubated at 37 ℃ for 4 h. Then acid-isopropanol was added to the wells and mixed thoroughly to dissolve dark blue crystals. Enzyme-linked immunosorbent assay was read at 570 nm[13]. Nitrite production by the neurons was measured by Griess assay. Briefly, an equal volume of Griess reagent (sulfanilamide 1 %, naphthylethylene diamine 0.01 %, H_3PO_4 3 %) was added to the conditioned media (without phenolsulfonphthalein), and the reaction was allowed to proceed at 22 ℃ for 10 min before the absorbance at 540 nm was measured. The concentration of nitrite ($[NO_2^-]$) was calculated by sodium nitrite as a standard[14,15].

Measurement of neuronal intracellular calcium Rat cortical cells were dissociated and prepared[16]. Cells were suspended in HEPES-Hanks (NaCl 137, $CaCl_2$ 1.3, $MgSO_4$ 0.4, $MgCl_2$ 0.5, KCl 5.0, KH_2PO_4 0.4, Na_2HPO_4 0.6, $NaHCO_3$ 3.0, glucose 5.6, HEPES 20.0 mmol·L^{-1}, pH 7.4) and loaded with Fura 2-AM (Sigma) at 37 ℃ for 45 min and collected by centrifugation at 1000 × *g* for 5 min. The loaded cells were washed twice and suspended in Mg^{2+}-free KRH solution. The $[Ca^{2+}]_i$ was determined from the ratio of fluorescence emission using two excitation wavelengths (350 nm and 380 nm) with AR-CM-MIC cation measurement system (Spex Industries Inc, USA). The system was calibrated according to: $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](F_D/F_S)$. The R_{max} and R_{min} were determined by calcimycin and egtazic acid, respectively.

Statistics All data were presented as $\bar{x} \pm s$ and compared by *t*-test.

RESULTS

Effects of *L*-PGA on Glu-induced neurotoxicity and NO release Twenty-four hours after cultures were exposed to Glu, the neuronal viability rate by trypanblau staining and the absorbance at 570 nm (A_{570}) by MTT assay were markedly decreased, indicating the apparent loss of living cells. In contrast, neurons in cultures exposed to the same amount of Glu in the presence of *L*-PGA appeared remarkably preserved and cleaved MTT. *L*-PGA 10 – 80 μmol·L^{-1} inhibited Glu-induced neuronal loss in a concentration-dependent manner with IC_{50} (41 ± 9) μmol·L^{-1} (95 % confidence limits: 30.3 – 54.7 μmol·L^{-1} (Tab 1).

Cortical cell cultures exposure to Glu 500 μmol·L^{-1} increased the formation of nitrite 2 times that of control group. When the cultures were pretreated with *L*-PGA, Glu-induced increment of nitrite was attenuated (Fig 1).

Tab 1. Protective effects of *L*-PGA on glutamate 500 μmol·L^{-1} (Glu)-induced neurotoxicity in primary rat cortical neurons. $n = 4$ culture wells per condition. $x \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* Glu group.

Groups	Concentration/ μmol·L^{-1}	A_{550}	Inhibitory rate/ %	Viability rate/ %
Control		2.13 ± 0.13		89.5
Glu		1.48 ± 0.29		28.3
Glu + *L*-PGA	80	1.97 ± 0.06^c	73.1	77.1
	40	1.85 ± 0.11^b	55.2	56.0
	20	1.63 ± 0.12^a	22.4	41.8
	10	1.51 ± 0.29^a	4.5	29.3
Glu + Ketamine	100	1.70 ± 0.46^b	32.8	47.5

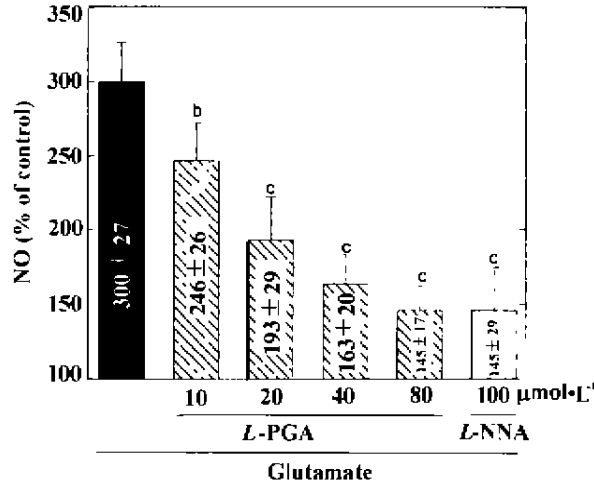

Fig 1. Effects of *L*-PGA on NO release in primary cortical neurons with Glu 500 μmol·L^{-1} only or pretreated with different concentrations (μmol·L^{-1}) of *L*-PGA or N^G-nitro-*L*-arginine (*L*-NNA). $n = 4$ culture wells per condition. $x \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* Glu group.

Effects of *L*-PGA on Glu-induced $[Ca^{2+}]_i$ elevation The resting $[Ca^{2+}]_i$ of rat cortical cells was (102 ± 22) nmol·L^{-1} in Mg^{2+}-free KRH containing calcium 2.0 mmol·L^{-1}. Glutamate 100 μmol·L^{-1} increased $[Ca^{2+}]_i$ about 110 %. The incubation with *L*-PGA attenuated $[Ca^{2+}]_i$ elevation caused by Glu (Tab 2).

DISCUSSION

"Sheng Mai San" has been extensively used in ischemic/hypoxic cardiovascular or cerebrovascular diseases. The content of *L*-PGA in "Sheng Mai San" injection is about 300 mg·L^{-1}. We suspected that *L*-PGA was one of bioactive constituents of "Sheng Mai San". The present study confirmed and extended the fundamental observations that *L*-PGA antagonized Glu receptors[9,10,17]. With the cell culture as a model system for quantitatively characterizing pharmacology of neuronal injury, our results demonstrated that 24 h after transient exposure of cortical cultures to Glu, most neurons progressively degenerated and were replaced by debris with significant loss of neurites (under phase-contrast microscopy) and reduction of neuronal number that failed to cleave MTT. Pretreatment with *L*-PGA improved neuronal survival, indicating its protection against Glu insult. The measurement of resting $[Ca^{2+}]_i$ in cortical neurons was consistent with the previous observation[12]. Glutamate triggered a marked elevation of $[Ca^{2+}]_i$ and NO release, which

Tab 2. Effects of *L*-PGA on Glu (100 μmol·L^{-1})-induced $[Ca^{2+}]_i$ elevation in rat cortical neurons. $n = 6$ experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Groups	Concentration/ μmol·L^{-1}	$[Ca^{2+}]_i$/nmol·L^{-1} Resting	Glutamate	$\Delta[Ca^{2+}]_i$	Inhibitory rate/ %
Control		102 ± 22	218 ± 20	115 ± 26	
L-PGA	1	105 ± 25	196 ± 29	91 ± 20^a	20.5
	3	102 ± 14	178 ± 12	75 ± 12^c	34.4
	10	120 ± 18	174 ± 20	60 ± 12^c	47.7
	30	102 ± 15	136 ± 16	34 ± 10^c	70.6
	100	93 ± 18	115 ± 18	22 ± 10^c	80.4
Ketamine	100	112 ± 13	177 ± 29	65 ± 16^c	57.5

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were largely attenuated by preincubation with *L*-PGA. Therefore, we speculated that the suppression of intracellular calcium accumulation and blockade of NO efflux might be related to the neuroprotection of *L*-PGA.

Taken together, our studies supported the hypothesis that by blocking Glu receptor-mediated intracellular calcium overload and the following cascade, especially overproduction of NO, *L*-PGA substantially reduced the vulnerability of cortical neurons to Glu insult.

REFERENCES

1 Smith-Swintosky VL, Mattson MP. Glutamate, beta-amyloid precussor protein and calcium mediated neurofibrillary degeneration. J Neural Transm 1994; 44 Suppl: 29 – 45.

2 Nicotera P, Ankarcrona M, Bonfoco E, Orrenius S, Lipton SA. Neuronal apoptosis versus necrosis induced by glutamate or free radicals Apoptosis 1996; 1: 5 – 10.

3 Yamauchi M, Omote K, Ninomiya T. Direct evidence for the role of nitric oxide on the glutamate-induced neuronal death in cultured cortical neurons Brain Res 1998; 780: 253 – 9.

4 Takaku T, Kameda K, Matsuura Y, Sekiya K, Okuda H. Studies on insulin-like substances in Korean Red Ginseng. Planta Medica 1990; 56: 27 – 30.

5 Qin B, Li XG, Zheng YN. Determination of pyroglutamic acid contents from different Ginsengs and different parts of Red Ginseng by HPLC method Chin Trad Herb Drugs 1994; 25: 75 – 6.

6 Drago F, Valerio C, D'Agata V, Astuto C, Spadaro F, Continella G, *et al*. Pyroglutamic acid improves learning and memory capacities in old rats. Funct Neurol 1988; 3: 137 – 43.

7 Mirzoyan SA, Zalinyan MG, Balasanyan MG, Topchyan A. Central vascular and metabolic effects of pyroglutamic acid Eksp Klin Farmakol (Russian) 1994; 57: 22 – 4.

8 Beni M, Pellegrini-Giampietro DE, Moroni F. A new endogeneous anxiolytic agent: *L*-pyroglutamic acid. Fundam Clin Pharmacol 1988; 2: 77 – 82.

9 Beani L, Bianchi C, Baraldi PG, Manfredini S, Pollini GP. Protection by pyroglutamic acid and some of its newly synthesized derivatives against glutamate-induced seizures in mice. Arzneimittelforschung 1990; 40: 1187 – 91.

10 de Mello CF, De La Vega DD, Pizutti LT, Lopes FP, Rubin MA, Homerich JG, *et al*. Neurochemical effects of *L*-pyroglutamic acid. Neurochem Res 1995; 20: 1437 – 41.

11 Giannola LI, De Caro V, Avellone G, Bousquet E. *L*-Pyroglutamyl-*L*-tryptophan derivatives as potential drug carriers. II. Permeation behaviour and stability in the gastro-intestinal tract. Pharmazie 1996; 51: 487 – 9.

12 Choi DW, Maulucci-Gedde MA, Kriegstein AR. Glutamate neurotoxicity in cortical cell culture. J Neurol Sci 1987; 7: 357 – 68.

13 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytoxicity assays. J Immunol Methods 1983; 65: 55 – 63.

14 Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J Immunol 1988; 141: 2407 – 12.

15 Akama KT, Albanese C, Pestell RG, Van Eldik LJ. Amyloid β-peptide stimulates nitric oxide production in astrocytes through an NFκB-dependent mechanism. Proc Natl Acad Sci USA 1998; 95: 5795 – 800.

16 Dildy JE, Leslie SW. Ethanol inhibits NMDA-induced increases in free intracellular Ca^{2+} in dissociated brain cells. Brain Res 1989; 499: 383 – 7.

17 Moret C, Briley M. The "forgotten" amino acid pyroglutamate. Trends Pharmacol Sci 1988; 9: 278 – 9.

L-焦谷氨酸对抗谷氨酸钠诱发的大鼠皮层神经元损伤

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关键词 吡咯烷酮羧酸；谷氨酸钠；钙；一氧化氮

目的：在大鼠皮层神经元研究 *L*-吡咯烷酮羧酸（*L*-PGA）对谷氨酸钠（Glu）诱发神经毒性的拮抗作用。**方法**：原代培养的皮层神经元取自 16 d 龄的胎鼠，与 Glu 作用 30 分钟，24 小时后测定神经元的存活及培养介质中亚硝酸盐的浓度；以 Fura 2-AM 为细胞内 $[Ca^{2+}]_i$ 荧光探针，AR-CM-MIC 阳离子测定系统测定 $[Ca^{2+}]_i$。**结果**：*L*-PGA 10 – 80 μmol·L^{-1}浓度依赖地抑制 Glu 500 μmol·L^{-1}引起的神经损伤，其 IC_{50}为（41 ± 9）μmol·L^{-1}，95 % 可信区间：（30.3 – 54.7）μmol·L^{-1}。*L*-PGA 也能浓度依赖地降低 Glu 引起的 NO 释放。*L*-PGA 1、3、10、30、100 μmol·L^{-1}对 Glu 100 μmol·L^{-1}引起的 $[Ca^{2+}]_i$ 升高的抑制率分别为 20.5 %、34.4 %、47.7 %、70.6 %、80.4 %。**结论**：*L*-PGA 可能通过抑制 NO 形成或细胞内 Ca^{2+} 浓度的升高而拮抗 Glu 的神经毒性。

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