Epigallocatechin gallate protects dopaminergic neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity by inhibiting microglial cell activation

LI Rui[1,2], PENG Ning[1], DU Fang[2], LI Xu-ping[2], LE Wei-dong[2]

[1]Department of Neurology, People's Hospital of Shaanxi Province, Xi'an 710068, China; [2]Health Science Center, Shanghai Institute for Biological Science, Chinese Academy of Science, Shanghai 200025, China

Abstract: Objective To observe whether the dopaminergic neuroprotective effect of (-)-epigallocatechin gallate (EGCG) is associated with its inhibition of microglial cell activation *in vivo*. **Methods** The effects of EGCG at different doses on dopaminergic neuronal survival were tested in a methyl-4-phenyl-pyridinium (MPP+)-induced dopaminergic neuronal injury model in the primary mesencephalic cell cultures. With unbiased stereological method, tyrosine hydroxylase-immunoreactive (TH-ir) cells were counted in the A8, A9 and A10 regions of the substantia nigra (SN) in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated C57BL/6 mice. The effect of EGCG on microglial activation in the SN was also investigated. **Results** Pretreatment with EGCG (1 to 100 μmol/L) significantly attenuated MPP+-induced TH-ir cell loss by 22.2% to 80.5% in the mesencephalic cell cultures. In MPTP-treated C57BL/6 mice, EGCG at a low concentration (1 mg/kg) provided significant protection against MPTP-induced TH-ir cell loss by 50.9% in the whole nigral area and by 71.7% in the A9 region. EGCG at 5 mg/kg showed more prominent protective effect than at 1 or 10 mg/kg. EGCG pretreatment significantly inhibited microglial activation and CD11b expression induced by MPTP. **Conclusion** EGCG exerts potent dopaminergic neuroprotective activity by means of microglial inhibition, which shed light on the potential use of EGCG in treatment of Parkinson's disease.

Key words: Parkinson disease; green tea polyphenols; microglia;1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; neuroprotection

Progressive chronic dopaminergic neuronal loss in the substantia nigra is the pathological hallmark of Parkinson disease (PD) characterized by rigidity, tremor, bradykinesia, and postural instability for unknown causes. Oxidative stress, local chronic inflammatory response, mitochondrial dysfunction and hereditary factors have been implicated in the pathogenesis of PD. Recent advances in etiological research of PD have directed attentions to microglial activation-induced neuronal injury, which may play a key role in the pathogenesis of PD [1], and preventing dopaminergic neuronal injury due to microglia-mediated inflammatory insults in the midbrain may prove to be fruitful means to slow down the process of dopaminergic neuronal loss. Green tea has long been used as a drug in traditional Chinese medicine for daily heath care and disease therapy, as described in classical works in ancient Chinese medical literatures. In recent years, green tea and its extracts have been shown to possess multiple pharmacological activities, and green tea polyphenol, an active component in green tea, has received much attention for its neuroprotective effects. Epidemiological findin gs provide strong evidence for the close association between regular drinking of green tea and lowered risk of PD [2], and in previous studies, green tea polyphenol was demonstrated to possess potent dopaminergic neuroprotective effect *in vitro* [3, 4]. We also found previously that (-)-epigallocatechin gallate (EGCG), a major monomer of green tea polyphenol, had direct inhibitory effect on microglial activation *in vitro* [5]. In this study, we further investigated the link between dopaminergic neuroprotection and microglial inhibition by EGCG.

MATERIALS AND METHODS

Animals and agents

Male C57BL/6 mice weighing 20-25 g provided by Experimental Animal Center of Chinese Academy of Science, Shanghai (Certification number: 99-003) were used in the experiment in line with the Care and Use of Laboratory Animals of Chinese Academy of Science and with approval by the Experimental Animal Center of Shanghai. DNase I, laminin, poly-L-lysine, EGCG (purity>95%), all-trans retinoic acid, and monoclonal antibody (mAb) against tyrosine hydroxylase (TH) were purchased from Sigma (St. Louis, MO). CD11b mAb was purchased from Chemicon. Dulbecco's modified

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LI Rui (1972-), MD, PhD, associate chief physician, Tel: 029-85251331-2561, E-mail: lrmail@yeah.net

Eagle's medium (DMEM), Ham's nutrient mixture F12 and other cell culture agents were from Invitrogen (Carlsbad, CA).

Primary mesencephalic cell culture

Primary mesencephalic cell culture was performed as described previously [5]. Briefly, the mesencephalic region was dissected from 14-day-old embryonic fetal rat brain and then minced and digested with trypsin (0.025%) and DNase I (0.01%). After mechanical dissociation by pipetting, the cells were washed twice with DMEM containing 10% fetal bovine serum (FBS) and seeded at a density of 1.5×10^5 per well in 96-well plates previously coated with poly-L-lysine. After cell culture for 48 h, the media were changed to serum-free medium for the experiment.

Animal grouping and treatment

Thirty male C57BL/6 mice were randomly assigned into 5 groups, with 6 in each group. The mice in the 3 EGCG treatment groups received intraperitoneal injection of EGCG (dissolved in normal saline) at 1, 5, and 10 mg/kg, respectively, before intraperitoneal 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration (for a total dose of 80 mg/kg given in 4 injections at a 2-hour interval) 7 days thereafter. An untreated control group and a MPTP-only group were included. The mice were sacrificed 12 days after MPTP injection and perfused with ice-cold normal saline for 10 min followed by perfusion with 4% formaldehyde for 3 h via the left cardinal ventricular. The brains were isolated and immersed in 20% sucrose overnight for immunohistochemical study.

Immunohistochemistry for dopaminergic neurons and microglial cells

For tyrosine hydroxylase (TH) immunocytochemistry, cultured primary mesencephalic cells were treated with 40 μmol/L methyl-4-phenyl-pyridinium (MPP^+) for 24 h, and some wells were pretreated with EGCG at different doses (1-100 μmol/L) 30 min before MPP^+ administration. The treated cells were then fixed with 4% formaldehyde for immunocytochemistry analysis. Briefly, the cells were incubated with anti-rat monoclonal antibody against TH (1∶6 000 dilution) overnight at 4 ℃ and then with anti-mouse antibody (1∶100) for 30 min at room temperature followed by visualization with ABC method. Positively stained cells in 10 random fields per well were counted for 8 wells and the data were collected from triplicate experiments.

To assess the protective effect of EGCG on dopaminergic neuron in the midbrain more accurately, we counted TH immunoreactive cells in the midbrain with unbiased stereological method as described previously [6].

The alternate sections were used for immunohistochemistry for CD11b, a specific microglial membrane antigen in the brain which expresses intensively activated microglial cells. CD11b antibody (1∶1 500) were added in the floating sections and incubated overnight at 4 ℃. The following steps of immunohistochemistry were similar with TH staining.

Western blot analysis for CD11b

The tissues of the substantia nigra (SN) region were lysed in RIPA lysis buffer (containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1 mmol/L PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). After measurement of the protein concentration by Bradford method, 20 μg of the protein underwent SDS-PAGE on 8% gel. The separated protein was then transferred onto nitrocellulose membrane and incubated overnight with anti-CD11 antibody (1:200) and detected with SuperSignal™ detection kit according to the manufacture's instruction. A duplicate sample was run on a 12% SDS-PAGE and then detected with β-actin antibody to serve as the internal control.

Statistical analysis

The data were expressed as *Mean*±*SE*. Differences between multiple groups were examined by one-way ANOVA following post-hoc test with SPSS 10.0 software. A *P* value less than 0.05 was considered to denote statistical significance.

RESULTS

Neuroprotection of EGCG on MPP^+-induced dopaminergic neuronal depletion in primary mesencephalic cell cultures

In comparison with the untreated control group, treatment with 40 μmol/L MPP^+ induced a marked reduction (75%) of TH-positive cells (Fig.1), which exhibited morphological changes of the neurite retraction, while pretreatment with EGCG at 1 μmol/L resulted in a 58.3% reduction of TH-positive cells. EGCG treatment at 10 μmol/L and 100 μmol/L showed

significant protection (at the rate of 80.5% and 58.3%, respectively) against MPTP-induced dopaminergic neuronal loss and preserved the cell structures (Fig.2 D, E, F).

Dopaminergic neuroprotection of EGCG in MPTP-treated C57BL/6 mice

MPTP injection of a total dose of 80 mg/kg caused a significant decrease in TH-positive cell number in the midbrain of C57BL/6 mice, and the reduction was the most obvious in the A9 region (45.8%). In A8 and A10 regions of the midbrain, 30.7% and 16.6% reduction of TH-positive cell number occurred, respectively. Pretreatment with EGCG at all doses effectively attenuated such neuronal loss by 50.9% in the total midbrain, and the dose of 5 mg/kg yielded the most prominent effect (Tab.1).

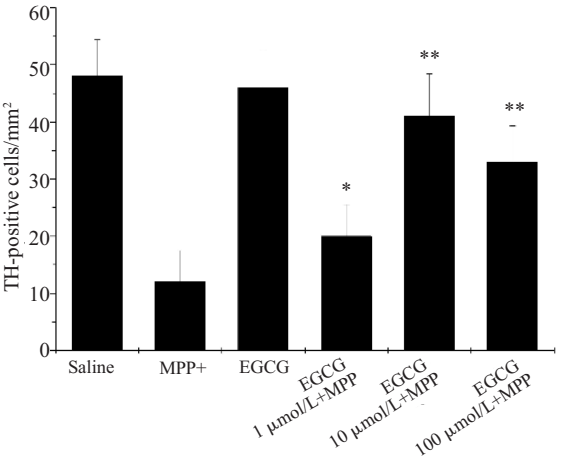

Fig.1 EGCG prevents dopaminergic cell from MPP⁺-induced death in primary mesencephalic culture

MPP⁺ induced a marked depletion of TH immunoreactive cells, while 1, 10 and 100 μmol/L EGCG attenuated MPP⁺-induced dopaminergic cell injury. EGCG alone did not affect the number of TH-positive cells.*P<0.05, **P<0.01 *vs* MPP⁺- treated group.

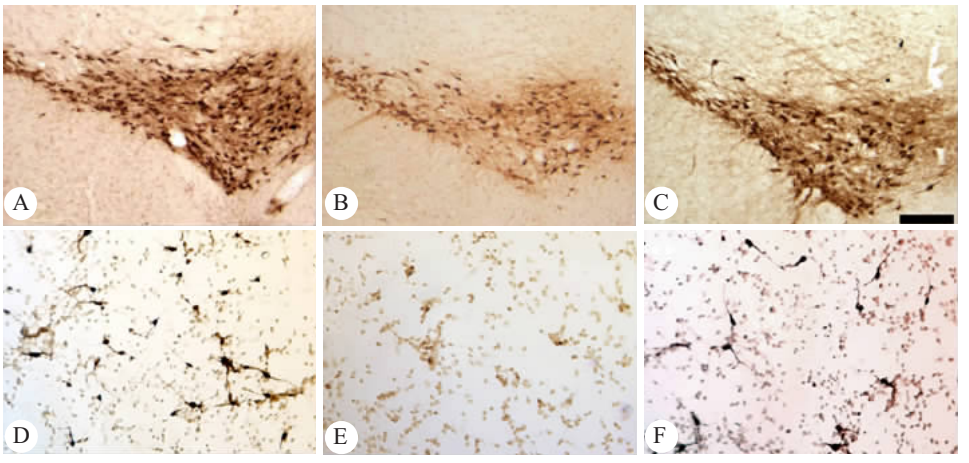

Fig.2 Neuroprotection of EGCG against MPTP-induced dopaminergic neuronal injury in C57BL/6 mouse midbrain and MPP+-induced primary mesencephalic culture.

TH immunohistologic staining showed that the total dose of 80 mg/kg MPTP caused a marked decrease of TH-positive cells in the substantia nigra (B) as compared with saline-treated group (A). Pretreatment with 5 mg/kg EGCG significantly reduced the decrease (C). In primary mesencephalic culture system, 40 μmol/L MPP+ decreased TH-positive cell number significantly (E) as compared with saline group (D), and 10 μmol/L EGCG pretreatment provided significant protection against TH-positive neuron depletion (F). Scale bar=100 μm.

Tab.1 Number of TH-positive neurons in the midbrain of C57BL/6 mice treated with various doses of EGCG (n=6)

Group	Midbrain areas			Total
	A8	A9	A10	
Saline	1361±45	4632±310	4018±133	9979±174
MPTP	943±36	2512±288	3350±114	6815±153
EGCG (1 mg/kg)+MPTP	1045±40*	4033±296**	3686±112*	8633±153**
EGCG (5 mg/kg)+MPTP	1178±56**	4125±337**	3878±143**	9177±156**
EGCG (10 mg/kg)+MPTP	963±49	3947±423**	3514±122*	8394±146*

*P<0.05, **P<0.001 *vs* MPTP group

Effects of EGCG on MPTP-induced microglial activation in the midbrain

Lightly stained CD11b-positive microglial cells were seen in midbrain in saline- treated mice, with tiny cell body and thin ramifications which characterized their resting status. MPTP administration resulted in the appearance of heavily stained, amoeboid CD11b-positive cells, with a marked increase in their number in the midbrain, suggesting the activation of the microglial cells in the

region. EGCG markedly attenuated the morphological changes of the activated microglial cells (Fig.3). Western blot analysis of CD11b also confirmed that EGCG dose-dependently inhibited MPTP-induced CD11b expression in the SN region in C57BL/6 mice (Fig.4).

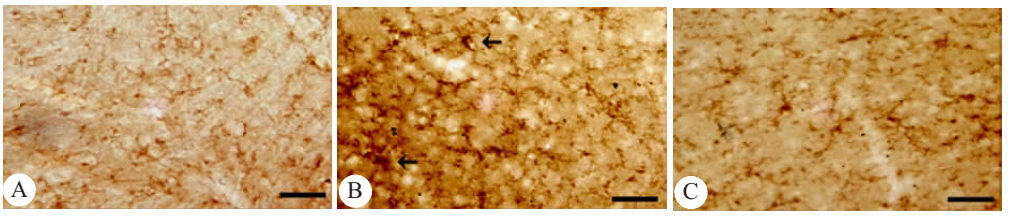

Fig.3 Inhibition of EGCG on MPTP-induced microglial activation in C57BL/6 mice

Saline treatment did not induce obvious microglia activation (A). MPTP administration caused a marked increase in CD11b-immunoreactive cells and enlargement of the microglial cell body (B). Pretreatment with EGCG partially inhibited the morphological change and reduced CD11b immunoreactivity in microglial cells (C). Arrow indicates CD11b-immunoreactive microglia. Bar=100 μm.

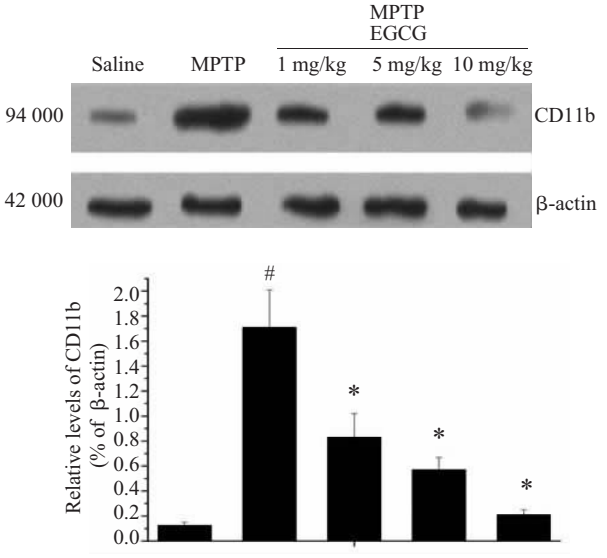

Fig.4 Western blot analysis of CD11b expression in the substantia nigra of C57BL/6 mice

Homogenates of the SN region were lysed with RIPA buffer and underwent 8% SDS-PAGE. CD11b was detected with anti-mouse mAb. MPTP treatment induced a marked increase of CD11b expression ($^{\#}P<0.001$), and EGCG dose-dependently attenuated the expression of CD11b in the SN ($^{*}P<0.05$).

DISCUSSION

The neuroprotective activity of EGCG *in vivo* was first reported by Levites *et al* [7]. The authors of the present paper believe that EGCG's neuroprotective activity is associated with its antioxidative capacity. Nevertheless, because microglial cells are the major contributors of oxidative stress and chronic inflammatory process, we hypothesized that the neuroprotective effects of EGCG are essentially through the inhibition of microglial activation. In this study, based on our previous finding that microglial activation could be significantly inhibited by EGCG, we tested the hypothesis in MPTP-induced dopaminergic neuronal injury model both in primary cultured mesencephalic cells and the midbrain of C57BL/6 mice and found that MPTP-induced microglial activation in the midbrain was significantly inhibited by EGCG administration, which establishes the close relation between dopaminergic neuroprotection by EGCG and the anti- inflammatory activity of EGCG.

In this study, we investigated the protective effects of EGCG on MPP^+-induced dopaminergic neuronal injury in primary mesencephalic cell cultures. The results demonstrated that small-dose EGCG (1 μmol/L) significantly ameliorated MPP^+-induced TH-positive cell depletion in primary mesphalic cell culture in a dose-dependent manner (within a certain dose range). Coincident with the findings of Levites, we found that EGCG at higher dose (100 μmol/L) did not produce stronger effect than 10 μmol/L EGCG, which seems to suggest a window of the concentration dependence of EGCG's action, and beyond this concentration range the effect of EGCG becomes detrimental to the neurons.

We demonstrated that peritoneal EGCG injection at the dose between 1 and 10 mg/kg significantly attenuated MPTP-induced TH-positive cell reduction in the SN, accompanied with a marked suppression of microglial activation. Microglial activation may not serve only as the defensive response against neuronal death, but also as the first event in the host following harmful insults to lead to the neuronal injury, since some neurotoxins such as MPTP and rotenone trigger glial activation before causing neuronal death[8,9]. Accompanying

microglial activation, excessive proinflammatory factors such as tumor necrosis factor (TNF)- and interleukin (IL)-1β as well as oxidative free radicals are produced by activated microglia, which may closely relate to neuronal injury [10]. Consequently, inhibition of microglial overactivation may favor neuronal survival. Hence, suppression of microglial activation has been generally recognized as one of the therapeutic approaches to some neurodegenerative diseases including PD. Together with our previous findings [5], the results of this study that EGCG inhibits microglial activation in vivo may lend support to the expansion of EGCG's potential use in the treatment of other neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis.

In summary, this study demonstrates that the neuroprotection of EGCG is closely associated with its capacity of inhibiting microglial activation, which offers evidence for the possible use of EGCG as a novel therapeutic agent for a wide variety of neurodegenerative diseases.

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表没食子儿茶素没食子酸酯的多巴胺能神经元保护作用

李 锐[1,2],彭 宁[1],杜 芳[2],李旭平[2],乐卫东[2](1 陕西省人民医院神经科,陕西 西安 710068;2 中科院上海生命科学研究院健康中心,上海 200025)

摘要:目的 研究绿茶多酚的主要单体成分表没食子儿茶素没食子酸酯(EGCG)的多巴胺能神经元保护作用。**方法** 利用 1- 甲基 -4- 苯基 -1, 2, 3, 6- 四羟吡啶(MPTP)及其代谢产物 1- 甲基 -4- 苯基吡啶(MPP+)分别造成选择性多巴胺能神经元损伤动物模型和细胞模型,给予不同剂量 EGCG,免疫组织化学染色方法标记酪氨酸羟化酶(TH)阳性细胞作为多巴胺能神经元标志,通过体视学方法观察不同剂量 EGCG 对阳性细胞数目的影响,同时利用膜抗原 CD11b 标记小胶质细胞,观察 EGCG 对小胶质细胞激活的作用。**结果** 在 MPP+ 诱导的原代培养中脑腹侧多巴胺能神经元损伤模型中,预先给予 EGCG 1 μmol/L~100 μmol/L 可显著减轻 MPP+ 诱导的多巴胺能神经元减少,保护率为 22.2%~80.5%。在体情况下,1 mg/kg 剂量 EGCG 腹腔注射对 MPTP 模型小鼠 A9 区 TH 免疫阳性细胞丢失的保护率为 71.7%,对中脑区域总 TH 免疫阳性细胞数丢失的保护率为 50.9%,5 mg/kg 和 10 mg/kg 剂量组中脑各区域 TH 阳性神经元数量均较模型组高($P<0.05$),同时,EGCG 对 MPTP 所致的中脑部位小胶质细胞的激活具有抑制效应。**结论** 本研究结果提示 EGCG 在一定剂量范围内对多巴胺能神经元具有显著的保护作用,具有潜在治疗帕金森病的价值,EGCG 对多巴胺能神经元的在体保护作用可能与其抑制小胶质细胞的激活密切相关。

关键词:帕金森病;绿茶多酚;1- 甲基 -4- 苯基 -1, 2, 3, 6- 四羟吡啶;神经保护;小胶质细胞

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