The Effect of Delayed Administration of Green Tea Polyphenol, (-)-epigallocatechin-3-gallate, on the Change of Putrescine Level and Hippocampal Neuronal Cell Damage after Transient Global Ischemia in Gerbil

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Objective : (−)-Epigallocatechin gallate (EGCG) 'a green tea polyphenol' is a potent antioxidant and known to reduce the free radical-induced lipid peroxidation. In our previous study, systemic administration of EGCG immediately after ischemia has been shown to inhibit the hippocampal neuronal damage in the gerbil model of global ischemia. Polyamines, especially putrescine (PU) is thought to be important in the generation of brain edema and neuronal cell damage associated with various types of excitotoxic neuronal injury. We investigate the effects of delayed administration of EGCG on the changes in polyamine levels and neuronal damage after transient global ischemia in gerbils.

Methods : To produce transient global ischemia, both common carotid arteries were occluded for 3 min with micro-clips. The gerbils were treated with EGCG (50mg/kg, i.p.) immediately or 2hr after ischemia. Putrescine levels were examined in the cerebral cortex and hippocampus 24 hours after ischemia using high per-formance liquid chromatography.

Results : PU levels in the cerebral cortex and hippocampus were increased significantly after the ischemia. The administrations of EGCG immediately after the ischemia attenuated the ischemia-induced increase of PU level, however, 2 hr delayed EGCG administration did not reduce the increase of PU level. EGCG administered immediately or 2 hr after ischemia significantly reduced neuronal damage in the hippocampal CA1 region, respectively.

Conclusion : These findings suggest that EGCG may has a promise in the management of stroke.

KEY WORDS : Global ischemia · Gerbil · Neuroprotection · Green tea · Polyphenol · (−)-Epigallocatechin gallate.

Introduction

The naturally occurring polyamines in mammalian cells are putrescine (PU), spermidine (SD), and spermine (SM) that play an essential role in the process of cellular growth, development, and differentiation[40,45]. Endogenous polyamines have multiple effects in the central nervous system and have been suggested to be neurotransmitters or neuromodulators[47]. Various kinds of stressful stimuli including stresses, seizures, excitotoxic conditions, and traumatic brain injuries increase the polyamines responses[2-4,17,18,30,33]. The changes in brain polyamine levels after brain ischemia have been studied[4,15,38,39] and polyamine, especially putrescine is thought to be important in the generation of brain edema, blood-brain barrier breakdown, and neuronal cell damage associated with various type of brain injury including brain ischemia and trauma. Strategies including the inhibition of polyamine metabolism have been reported to have neuroprotective effect against ischemic neuronal injury[4,15,22,23].

The chemical composition of green tea contains many polyphenolic compounds, generally known as catechins. The main catechins in green tea are (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG). Among them, EGCG is the most active major polyphenol of green tea and primarily responsible for the green tea effect. In addition, EGCG has been demonstrated to display a potent antioxidant properties[18]. EGCG possesses two triphenolic groups in its structure, which are thought to be important for its stronger

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antioxidant action[34]. It is important to note that EGCG acts as an antioxidant in biological systems. Although the specific mechanisms of its antioxidant actions remain unclear, several pharmacological antioxidant properties of EGCG have been identified such as : (a) free radical scavenging activity or attenuation of lipid peroxidation due to various forms of radicals[19,26,42] ; (b) inhibition of xanthine oxidase activity[1] ; and (c) blockade of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) induction[9,32]. These action mechanisms may contribute to the potent antioxidant and putative neuroprotective actions of EGCG.

Oxygen free radical-induced lipid peroxidation has been strongly suggested to play an important role in the pathogenesis of delayed neuronal damage after global ischemia[21]. Using in vitro and in vivo models, recent studies suggest the protective effects of green tea extract and EGCG on neuronal damage induced by free radical attack[19,31]. In previous study, we reported systemic administration of EGCG reduced neuronal damage following transient global ischemia[29]. In the present study, we examined whether EGCG reduces PU level changes in brain regions and neuronal damage in the gerbil hippocampus after transient global ischemia.

Materials and Methods

Animals

Male Mongolian gerbils (*Meriones ungiculatus*) weighing 60−80g were used in this study. These animals were housed in laboratory cages and maintained on a 12-hour light-dark cycle, with *ad libitum* access to food and water throughout the study period. The gerbils were treated with EGCG (50mg/kg, i.p., purchased from Sigma Chemical Co., St. Louis, MO, USA) immediately or 2 hours after ischemia. EGCG was dissolved in normal saline. In the ischemic control groups, the vehicle (normal saline, i.p.) was administered immediately or 2 hours after ischemia. In this study, we used 62 gerbils totally and the animals were divided according to the experimental groups as follows. (1) sham-operated group : all of procedures were same with other except arterial occlusion (n=10, 5 for polyamine assay and 5 for histology). (2) vehicle 0 group : ischemic damaged group treated with vehicle immediately after ischemia (n=12, 6 for polyamine assay and 6 for histology). (3) EGCG 0 group : ischemic damaged group treated with EGCG immediately after ischemia (n=13, 5 for polyamine assay and 8 for histology). (4) vehicle 2 group : ischemic damaged group treated with vehicle 2 hours after ischemia(n=12, 7 for polyamine assay and 5 for histology). (5) EGCG 2 group : ischemic damaged group treated with EGCG 2 hours after ischemia (n=15, 6 for polyamine assay and 9 for histology).

Surgery

The gerbils were anesthetized with chloral hydrate (400mg/kg, i.p.). In the supine position, a midline ventral incision of 2cm was made in the neck. Both common carotid arteries were exposed, separated carefully from the vagus nerve, and occluded for 3 minutes with micro-clips. Blood flow during the occlusion and reperfusion after removal of the clips was confirmed visually and the incision was closed. The rectal temperature was monitored and maintained at 37±0.5℃ with a feedback-controlled heating pad (CMA, Stockholm, Sweden) and an incandescent light was placed over the head from the induction of anesthesia until 3 to 4 hours after ischemia and placed in warm box (at about 30℃) for 3 hours to avoid the biased results by hypothermia[28]. In the sham group, the neck incision was made only to expose both common carotid arteries without occlusion. Other procedures were identical to those of other groups.

Polyamine extraction and high performance liquid chromatography(HPLC) analysis

The animals were sacrificed 24 hours after ischemia for polyamine extraction[4,39]. The brains were removed rapidly from the skull and dissected into cerebral cortex and hippocampus. The extraction procedure was carried out in ice-chilled conditions. Derivation and HPLC analysis of polyamines were based upon the method of Spragg and Hutchings[43] with some modification. Each brain sample was homogenized with a glass tissue homogenizer in 10 volumes of ice-chilled 0.4M perchloric acid containing 2mM disodium EDTA and 1,8-diaminooctane 4×10^{-5}M as an internal standard. The homogenate was centrifuged at 12,000g for 10 minutes at 4℃ and 100 μl of the supernatant was evaporated by a vacuum drier. The dried tissue was dissolved in 100 μl of 1M sodium bicarbonate then deprived with 300 μl of 4-fluoro-3-nitrobenzotrifluoride (FNBT) reagent (a mixture of 10 μl of FNBT and 1ml of dimethyl sulfoxide) at 60℃ for 20 minutes. At the end of derivation, 40 μl of 1M histidine in 1M sodium bicarbonate was added to the reaction mixture then the derivation continued for another 5 minutes to scavenge excess FNBT. After cooling the mixture in an ice basket, the N-2-nitro-4-trifluoromethylphenyl derivatives of polyamines were extracted twice with 2ml of 2-methylbutane. After centrifugation at 3,000g for 10 minutes, the organic phase was evaporated under nitrogen gas flow and the residue was reconstituted with 1.0ml of HPLC grade methanol. The 20 μl of the methanol solution was applied to the isocratic reversed phase HPLC system (Gilson Medical Electronics, Villiers-le-

Bel, France), then the separation of NTP-polyamines was accomplished by elution of acetonitrile-water (85 : 15, v : v) mobile phase at the flow rate of 1.0ml/min within 30 min. The eluent was monitored by UV/VIS detector set at 242nm and a Microsorb^{TM} C18 column (5 μM, 4.6mm×25cm, Rainin instrument Co. Woburn, Mass, USA) was used[30].

Histology

The gerbils were sacrificed 5 days after ischemia. They were deeply anesthetized with diethyl ether and perfused transcardially with cold heparinized phosphate-buffered saline (PBS, pH 7.2) and 10% formalin in PBS. The brains were removed from the skull and fixed in the same fixative for 24 to 48 hours. Thereafter the brains were embedded in paraffin and representative coronal sections (6-μm thick), which included the dorsal hippocampus, were obtained with a rotary microtome. Tissue sections were stained with hematoxylin and eosin. A blinded investigator performed the histological examination. The hippocampal CA1 damage was determined by counting the surviving pyramidal neurons[27]. The mean number of CA1 pyramidal neurons per millimeter for both hemispheres in a section of dorsal hippocampus was calculated for each group of the gerbils.

Statistics

Statistical analysis was performed using ANOVA followed by Scheffe's post-hoc test and significance refers to results where $p<0.05$ was obtained.

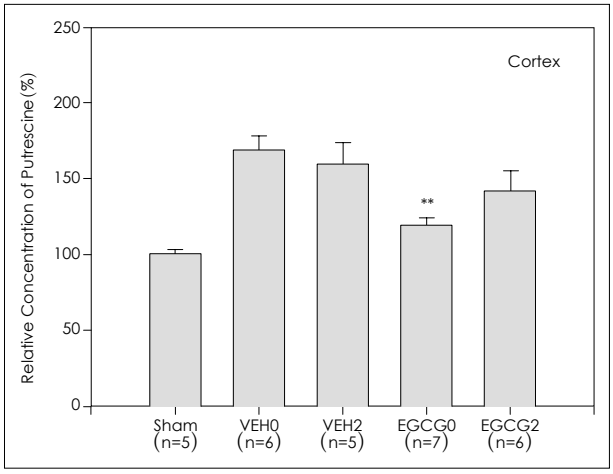

Fig. 1. Changes of putrescine (PU) levels in gerbil cerebral cortex after global ischemia. Sham, sham-operated (n=5); VEH0, vehicle-treated immediately after ischemia (n=6); VEH2, vehicle-treated 2 hours after ischemia (n=5); EGCG0, group treated with EGCG immediately after ischemia (n=7), and EGCG2, group treated with EGCG 2 hours after ischemia (n=6). Data expressed as mean±SEM. **p<0.01 for the comparison between group treated with EGCG and VEHO.

Results

Effect of delayed administration of EGCG on the changes in PU levels

The changes in polyamine levels were examined 24 hours after ischemia. The PU levels of the cerebral cortex increased after ischemia compared with sham-operated group (Fig. 1). In the hippocampus, the PU levels also increased after ischemia when compared to the sham-operated group (Fig. 2).

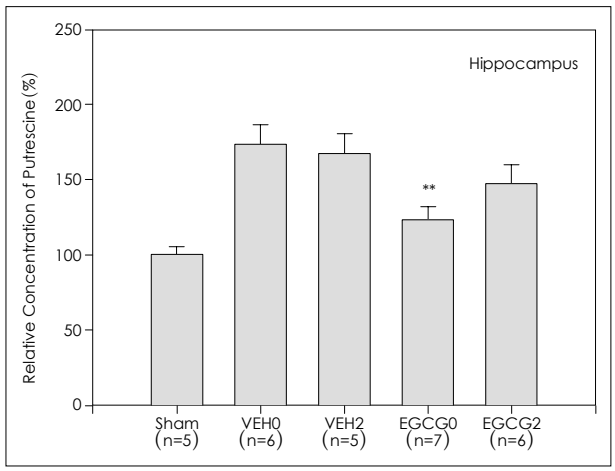

Fig. 2. Changes of putrescine (PU) levels in gerbil hippocampus after global ischemia. Sham, sham-operated (n=5); VEH0, vehicle-treated immediately after ischemia (n=6); VEH2, vehicle-treated 2 hours after ischemia (n=5); EGCG0, group treated with EGCG immediately after ischemia (n=7), and EGCG2, group treated with EGCG 2 hours after ischemia (n=6). Data expressed as mean±SEM. **p<0.01 for the comparison between group treated with EGCG and VEHO.

EGCG administered immediately after ischemia attenuated the increases of the cortical and hippocampal PU levels (respectively $p<0.01$, Fig. 1 and 2). However, EGCG administered 2 hours after ischemia failed to attenuate the increases of the cortical and hippocampal PU levels (Fig. 1 and 2).

Histology

Histological examination of the nervous system demonstrated marked cell damage in the hippocampal CA1 region in the gerbils treated with a vehicle when compared with the sham-operated group. CA1 pyramidal neurons showed pyknosis, eosinophilia, karyorrhexia, and chromosome condensation in the vehicle-treated group (Fig. 3). This neuronal cell damage was suppressed by EGCG administration. EGCG administered immediately or 2 hours after ischemic insult significantly reduced neuronal damage ($p<0.001$ and $p<0.001$, respectively, Fig. 4).

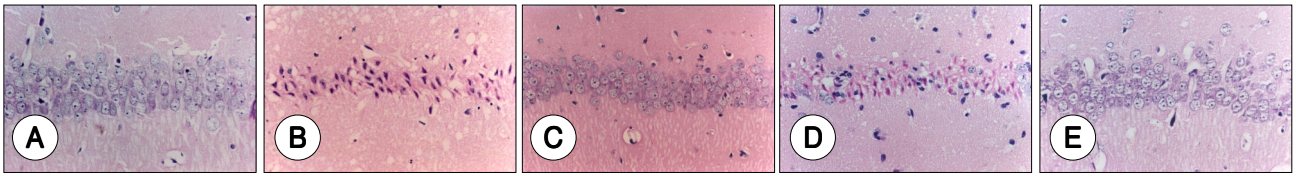

Fig. 3. Microphotographs of the hippocampal CA1 region in the gerbil 5 days after global ischemia(hematoxylin and eosin staining). CA1 region in sham-operated(A), vehicle-treated immediately after ischemia(B), vehicle- treated 2 hours after ischemia(C), EGCG-treated immediately after ischemia(D), and EGCG-treated 2 hours after ischemia(E). After ischemic insult, only a few normal cells are seen with round cell bodies and clear nuclei and nucleoli. Damaged cells are shrunken and distorted, with small dense nuclear remnants. Bar=50 μm.

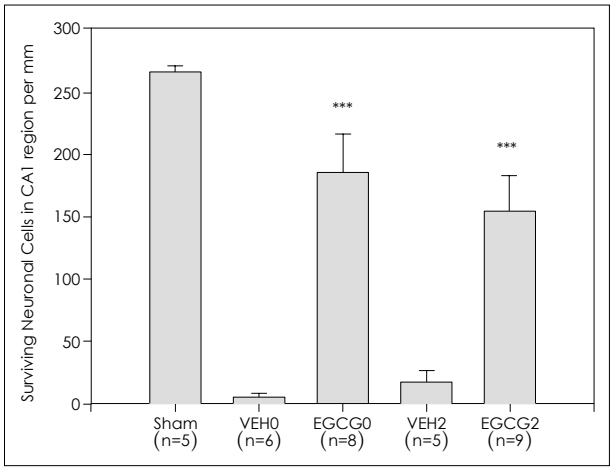

Fig. 4. Effect of EGCG on the number of surviving cells in the hippocampal CA1 region in the gerbil 5 days after global ischemia. Sham, sham-operated(n=5); VEH0, vehicle-treated immediately after ischemia(n=6); VEH2, vehicle- treated 2 hours after ischemia(n=8); EGCG0, group treated with EGCG immediately after ischemia(n=5), and EGCG2, group treated with EGCG 2 hours after ischemia(n=9). Data are expressed as mean±SEM. ***$p<0.001$ for the comparison between group treated with EGCG and their corresponding vehicle-treated groups, respectively.

Discussion

It is suggested that polyamines released from necrotic neurons into the extracellular compartment bind to the NMDA receptor of cells located in close vicinity and thus render neurons vulnerable to subtoxic levels of excitotoxins. Several researchers examined the changes in brain polyamine levels after focal or global ischemia[4,15,39]. Various kinds of stimuli or stresses such as seizures, excitotoxicity, and traumatic brain injury modify the ornithine decarboxylase(ODC), the regulatory enzyme in the polyamine biosynthesis[2,3,8,16,33]. These changes may be related to modifications of intracellular calcium ion fluxes because polyamines increase the cytosolic amino acids. Some authors have shown discrepancies between ODC activity and the concentration of polyamine[46], a finding suggesting that the latter might be more useful than the former.

In this study, PU levels in cortex and hippocampus increased after transient global ischemia. These changes in PU levels bear a strong similarity to those described by Paschen et al[39]. The diamine precursor of polyamines, PU is normally in low level and long lasting accumulation of PU may be harmful[39]. An association between brain damage and high PU levels in the ischemic brain has also been found previously suggesting a role for PU in mediating the ischemic damage. ODC and polyamines are thought to be important in the generation of edema and neuronal cell loss associated with cerebral ischemia[39]. Baskaya et al[3] suggested that polyamines may play a role in posttraumatic brain edema formation particularly in brain regions.

Polyamines are known to increase cytosolic calcium ion concentration[20,24,25] and induce the release of excitatory amino acid[8]. A remarkable increase of the extracellular concentration of excitatory amino acids including glutamate, induced by cerebral ischemia leading to a large amount of calcium ion influx through glutamate receptor in neurons and neuronal injury[6,12,36]. PU levels particularly correlate with the density of cell necrosis[39]. PU might be a reliable marker for acute pathology in brain tissue injury[35]. Tissue PU increased in the penumbra region that developed brain edema in permanent focal cerebral ischemia[4]. In addition, the blockade of ODC resulted in a protective effect against focal or global ischemic brain damage[22] and partially antagonized the convulsant activity[13] suggesting that polyamine metabolism plays a role in the development of neuronal injuries following brain ischemia or epileptic seizure. In regarding the effect of EGCG on the PU level, although there is no definite evidences, we can suggest two possibilities. First, EGCG attenuates the harmful accumulation of PU by influence on the polyamine metabolism. Second, EGCG-induced neuroprotection due to potent antioxidant effect may decrease the PU response to excitotoxicity.

In this study, SD and SM levels in the cortex and hippocampus showed no significant changes after ischemia and EGCG did not show any influences on the SD and SM levels (data not shown). Activation of interconversion pathway enzymes, SD/SM N_1-acetyltranseferase[48] and PA oxidase[5]

which convert SM to SD and SD to PU, is a probable major factor in PU accumulation[41]. Tissue PU levels change to a remarkable degree than those of SD and SM after various pathological conditions[2-4,14,24,25,30,38].

The results of this study suggest that administration of EGCG immediately after ischemia can reduce the accumulation of PU levels. However, 2 hours delayed administration of EGCG did not reduce PU accumulation. It seemed that polyamine biosynthesis increased rapidly after ischemia. Therefore, EGCG of delayed administration failed to reduce the ischemia-induced PU accumulation. However, the role of EGCG in inhibition of PU level or polyamine metabolism is not clear in this study and needs to be further studied.

In the present study, the delayed administration of EGCG reduced hippocampal pyramidal neuronal cell damage following global ischemia. Many researchers have described the effectiveness of green tea in inhibition of carcinogenesis, inflammation, and free radical-induced injury. Recently, the antioxidant effects of EGCG were extensively studied. EGCG shows protective effects against oxidative stress-induced lipid peroxidation in synaptosome[19] and the scavenging effect of peroxyl radicals[26]. Chiou et al[10] reported EGCG-induced facilitation of retinal function recovery after retinal ischemia. We reported the protective effect of EGCG against beta-amyloid protein-induced neuronal damage[11]. In addition, green tea extract has been shown to protect the liver, kidney and brain from lipid peroxidation injury[31,42]. EGCG has been demonstrated to pass the blood brain barrier and reach brain parenchyma in animal study[44]. In previous study, we reported protective effect of EGCG against neuronal damage induced by transient global ischemia in the gerbils when administered immediately after ischemic insults[29]. In this study, we tried EGCG administration at 2 hours after ischemia. In addition to EGCG treated immediately after ischemia, animals treated EGCG 2 hours after ischemia displayed a significant increase in the number of surviving neurons in the hippocampal CA1 region.

It has been well known that oxygen radical-induced lipid peroxidation has been strongly suggested to play a role in postischemic neuronal damage[9,21]. Recently, a variety of studies have examined the neuroprotective properties of antioxidants in brain ischemia[7,9,37]. Green tea contains many polyphenolic antioxidants and EGCG is the key polyphenolic antioxidant responsible for cancer chemoprevention, anti-inflammation, and neuroprotection. Although the mechanisms underlying the neuroprotective effect of EGCG are not fully understood, in summary, this study demonstrated that EGCG has a neuroprotective effect against hippocampal neuronal damage in a gerbil model of global ischemia.

Conclusion

The present results show that the administrations of EGCG early after ischemia can inhibit the transient global ischemia-induced increase of PU levels in brain regions. EGCG is neuroprotective against neuronal damage even when administered up to 2 hours after global ischemia. Because 2 hours delayed administration of EGCG failed to reduce PA level, PU may partially attribute to neuronal damage process in ischemia. However, the role of polyamines, especially PU, in the pathogenesis of brain ischemia is not clear and needs to be further studied. It seems that the potent antioxidant effects of EGCG contributed to its neuroprotective effect in this study. These findings suggest that EGCG may have a promise in the acute treatment of stroke.

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