Potential protection of green tea polyphenols against intracellular amyloid beta-induced toxicity on primary cultured prefrontal cortical neurons of rats

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A B S T R A C T
The present study was performed to explore the effect of green tea polyphenols on the intracellular Aβ (iAβ)-induced toxicity to cultured rat primary prefrontal cortical neurons. Administration of 100 nM, 1 μM or 10 μM of green tea polyphenols significantly inhibited the iAβ-induced toxicity to cultured rat primary prefrontal cortical neurons tested by MTT and LDH release assay. It further studied the involvement of neuroprotective pathway protein AKT in green tea polyphenols protection against iAβ-induced cytotoxicity on cultured rat primary prefrontal cortical neurons. The results demonstrated that the content of p-AKT decreased significantly after iAβ treatment, while administration of green tea polyphenols significantly inhibited the iAβ-induced decrease in the content of p-AKT. Moreover, blockade of AKT signalling inhibited the protective effects of green tea polyphenols against iAβ-induced neurotoxicity. The results suggest that green tea polyphenols may play a protective effect on cultured rat primary prefrontal cortical neurons against iAβ-induced cytotoxicity and AKT is involved in the green tea polyphenols-induced protective effects.

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It is well known that amyloid beta peptide (Aβ) is related to Alzheimer’s disease (AD) and the toxicity of extracellular Aβ has been well studied [6,13,14,17]. However, intracellular Aβ (iAβ) accumulation and toxicity have attracted attention lately [17]. Studies showed that accumulation of iAβ is an early event of AD, preceding the formation of extracellular Aβ [8,14,23]. Moreover, iAβ causes cell death in primary cultured neurons [22,26,33]. These results suggested that iAβ is a viable alternative candidate for explaining neuronal functional physiology and neuronal cell loss in the development of AD since the toxicity of extracellular Aβ is not consistent in some studies [17]. Therefore, searching for drugs or natural products targeting iAβ toxicity may potentially benefit for AD treatment.

Green tea is a popular beverage and is used widely in the world. It is known that green tea has many beneficial effects to human body, such as antimutagenic, antiproliferative, anticarcinogenic properties and neuroprotective activity in degenerative disorder models [1,5,10,16,28]. Polyphenols are the main compounds of green tea and there are a class of polyphenolic flavonoids known as catechins (the most abundant component) which comprise of (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG) and (−)-epicatechin (EC) respectively [32]. EGCG is thought to be the most pharmacologically active of the catechins. Studies have demonstrated that green tea polyphenols protected against ultraviolet irradiation induced injury in cultured neurons [19] and green tea polyphenols reduced hippocampal neuronal damage induced by ischemia [24].

The present study was performed to explore the influence of green tea polyphenols on the intracellular Aβ (iAβ)-induced toxicity to cultured rat primary prefrontal cortical neurons.

Primary cultured prefrontal cortical neurons of rats were prepared as previously described [25,26]. Every measure was taken to minimize pain of animals and experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1966. Newborn (postnatal day 0) Sprague-Dawley rat pups were provided by the Experimental Animal Center of Peking University Health Science Center (Beijing, China). Brain tissues were dissected in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA). The prefrontal cortex tissues were dissociated by mechanical chopping 10–20 times, and then dissociated with 0.25% trypsin (Invitrogen, Carlsbad, CA) for 30 min at 37 °C and filtered through nylon meshes to obtain a single-cell suspension. Cells were sedimented and resuspended in DMEM containing 10% fetal bovine serum, 2 g/L HEPES, penicillin (0.1 g/l), and streptomycin (0.1 g/l, all from Invitrogen, Carlsbad, CA). Cells were seeded in poly-L-lysine-coated petri dishes or plates and maintained in an atmosphere of 5% CO2 and 95% O2 humidified air at 37 °C. Cytosine

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arabinoside (10 μM; Sigma, St. Louis, MO) was supplemented after plating for 3 days to inhibit glia cell growth. Neurons were treated after 5 days of culture. The purity of neurons is 90% as described in our previous literature [25].

The green tea polyphenols (98% purity, a gift from Dr. Bao-Lu) contain of 50% EGCG, 22% ECG, 18% EGC, and 10% EC, analyzed by high-pressure liquid chromatography as described before [19].

Intracellular Aβ (1-42) cDNA was subcloned from pEGFP-N3 into pAdTrack with BglII and XhoI digestion. The adenovirus was packaged in HEK293 cells and the infectious particle was measured as 2 × 10^8/ml (MOI = 1.33). The purified virus supernatant was added to cell culture medium at DIV5 and we used EGFP virus transfection as control group.

The viability of cells after various treatments was estimated in terms of their ability to reduce the dye methyl thiazolyl tetrazolium (MTT, Sigma, MO) to blue formazan crystal. Primary rat prefrontal cortex neurons cultured in 96-well plate were gently washed with 0.01 M PBS. After washing, 90 μl of medium with 10 μl of MTT-PBS solution (5 mg/ml) was added to each well and the plate was maintained at 37°C for 2–4 h. Then the products were dissolved in DMSO for quantification by measuring the absorption at 570 nm using a micro-plate spectrophotometer (Bio-Rad CA), representing relative cell viability.

Cell cytotoxicity after various treatments was evaluated by LDH release. This was achieved with a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit according to the manufacturer’s instructions (Promega, Madison, WI).

Protein lysates of prefrontal cortex neurons were extracted after treatment. Neurons cultured in 6-well plates were washed three times with 0.01 M PBS, after which, 100 μl of lysis buffer with 1 mM phenylmethanesulfonyl fluoride (PMSF) was added into each well and the plate was maintained at 37°C for 2–4 h. Then the products were dissolved in DMSO for quantification by measuring the absorption at 570 nm using a micro-plate spectrophotometer (Bio-Rad CA), representing relative cell viability.

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Moreover, chronic administration of green tea polyphenols significantly reversed scopolamine-induced retention deficits in both step-through passive avoidance and spontaneous alternation behavior tasks [12]. In vitro, green tea polyphenols protected against extracellular Aβ-induced neurotoxicity in primary cultured hippocampal neurons [3,7]. Here, we show that green tea polyphenols significantly inhibited virus-mediated Aβ-induced neurotoxicity in primary cultured prefrontal cortical neurons. These data are consistent with the hypothesis that green tea polyphenols may be beneficial according to the amyloid hypothesis of Alzheimer’s disease.

The concentrations of EGCG (750–1255 µg/ml or 165–275 µM) and in ECG (150–361 µg/ml or 34–82 µM) present in green tea [3] are higher than those required to produce protective effects in the present study (from 100 nM to 10 µM). Numerous studies have reported polyphenols-mediated neuroprotection, there is little information about the penetrability of polyphenols or their metabolites with the blood–brain barrier (BBB) [29]. Although most polyphenols are rapidly metabolized in human body, a study has shown that isotope labeled EGCG (33% of the total administered radioactivity) was found in the mouse brain after a single peripheral administration of [3H]EGCG [30]. Moreover, studies showed that peripheral administration of EGCG (50 mg/kg, i.p.) had neuroprotective effects in models of ischemia [3]. These results are consistent with the present study. Therefore, further studies involving green tea polyphenols are needed.

**Fig. 1.** Influence of green tea polyphenols on Aβ-induced neurotoxicity in primary cultured prefrontal cortical neurons. (A) Inhibition of green tea polyphenols on Aβ-induced neurotoxicity in primary cultured prefrontal cortical neurons tested by MTT assay. (B) Influence of green tea polyphenols alone on cell viability in primary cultured prefrontal cortical neurons tested by MTT assay. (C) 100 nM, 1 µM or 10 µM of green tea polyphenols reduces Aβ-induced cytotoxicity in the neurons assessed by LDH release assay. * is referring to compare to the control group, # is referring to compare to the Aβ group.

**Fig. 2.** Involvement of AKT in the protective effects of green tea polyphenols in the neurons. (A) Green tea polyphenols restores Aβ-induced decreases in the content of p-Akt in the cultured neurons. Top panel, representative Western blot probed with an antibody specific against p-Akt after various treatments in the neurons. Bottom panel, summary of optical density of p-Akt. * is referring to compare to the control group. # is referring to compare to the Aβ group. (B) AKT inhibitor blocked the protective effects of green tea polyphenols against Aβ-induced neurotoxicity in neurons as assessed by MTT assay. * is referring to compare to Aβ with polyphenols group.
conducting on animal models of AD or on patients with Alzheimer’s disease.

Studies have demonstrated that Akt is involved in the progression of AD [15,22]. Activation of Akt positively regulates the anti-apoptotic protein bcl-2 [4] and negatively regulates the pro-apoptotic protein bax [27]. Upon apoptosis, Bax undergoes a conformational change and then it is translocated to mitochondria, leading to the release of cytochrome c and the activation of caspase [6]. Our previous work showed that iAβ caused inactivation of Akt and activation of caspase-3 in primary cultured neurons and curcumin inhibited the effects [26]. The present work showed that polyphenols rescued the iAβ induced inactivation of Akt, it is very likely that polyphenols inhibited the activation of caspase-3 by iAβ. Green tea averts age dependent decline of hippocampal signaling system including bcl-2 which related to antioxidative defenses and survival [2]. More recently, a study showed that polyphenols inhibited the irradiation induced bax activation [20]. These results support our hypothesis that Akt-bax (bcl-2)-caspase-3 signaling pathway may be involved in the neuroprotective effects of polyphenols. More importantly, our present data demonstrated that the Akt inhibitor LY29004 blocked the protective effects of green tea polyphenols and previous studies showed that LY29004 alone did not have any effects on cell viability in primary cultured neurons [18]. This study further confirms the important role of Akt in the protective effects of green tea polyphenols in iAβ induced neurotoxicity in AD. Interestingly, several studies demonstrated that polyphenols prevented the aggregation of amyloid beta and this at least in part mediated the neuroprotective roles of polyphenols [3,9,31]. We hypothesized that green tea polyphenols prevented the aggregation of iAβ, thus normalized the dysfunction of neuroprotective Akt signaling pathway caused by iAβ and produced neuroprotective effects in the neurons.

In conclusions, results of the present study demonstrate that green tea polyphenols has a protective effect against iAβ-induced neurotoxicity, indicating a new potential therapeutic intervention of green tea polyphenols in the progress of Alzheimer’s disease.

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