Food and Chemical Toxicology 49 (2011) 2618–2623

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Epigallocatechin-3-gallate protects rat brain mitochondria against cadmium-induced damage

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ARTICLE INFO

Article history:
Received 19 October 2010
Accepted 7 July 2011
Available online 26 July 2011

Keywords:
Epigallocatechin-gallate
Cadmium ion
Neurotoxicity
Mitochondrial function

ABSTRACT

Many health claims have been made about the medicinal benefits of drinking green tea, including neuroprotection. This study mainly focuses on Epigallocatechin 3-gallate (EGCG), a potent antioxidant, which is abundantly found in green tea. Cadmium [Cd^{2+}] is a toxic pollutant that leads to neurotoxicity in both animals and humans. Although the entrance of Cd^{2+} in the adult central nervous system is limited, developmental neurotoxicity has been evidenced as result of the blood–brain barrier (BBB) immaturity. Moreover, high Cd^{2+} levels are known to impair BBB function. Furthermore, the molecular mechanisms related to its neurotoxic properties remain unknown. This study evaluates the potential protective effect of the major green tea polyphenol, EGCG, against Cd^{2+}-induced mitotoxicity under *in vitro* conditions, using mitochondrial-enriched fractions from rat brain. Co-incubation of EGCG with Cd^{2+} prevented the Cd^{2+}-induced mitochondrial dysfunction (capacity to reduce MTT to formazan). In addition, EGCG completely prevented mitochondrial lipid peroxidation induced by Cd^{2+} but did not affect non protein thiols levels. Spectroscopic studies have shown EGCG able to form a chemical complex with Cd^{2+}, in an equimolar ratio. In this study we demonstrate EGCG effectiveness in protecting against Cd^{2+}-induced mitochondrial dysfunction and lipid peroxidation probably due to its antioxidant and chelating effects.

1. Introduction

Many health claims have been made about the medicinal benefits of drinking green tea, including neuroprotection (Weinreb et al., 2009). This study mainly focuses on Epigallocatechin-3-gallate (EGCG), a potent antioxidant, which is abundantly found in green tea.

The chemical structures of catechins contribute to their antioxidant properties. Some catechins, such as EGCG, have a gallate moiety esterified at the 3rd position of the C ring, the catechol group on the B ring and the hydroxyl groups at the 5th and 7th positions on the A ring (Fig. 1). The potent free radical scavenging activity of EGCG was attributed to the presence of the gallate group (Devika and Stanely Mainzen Prince, 2008).

It is important to mention that polyphenol compounds (including green tea catechins) have metal chelating properties (Hider et al., 2001; Fernandez et al., 2002), which have been attributed mainly to their gallate group (Kumamoto et al., 2001).

Cadmium (Cd^{2+}), a highly toxic heavy metal, is mainly released from the burning of fossil fuels and municipal wastes, refining of metals and consumption of tobacco product, resulting in the pollution of water, air, and soil (Chen et al., 2008). Once it is widely distributed and extensively used in modern society, human exposure to this and other toxic metals is unavoidable (Pourahmad et al., 2003).

Furthermore, natural systems are complex and many factors influence the bioavailability of Cd^{2+}. Changes in pH, temperature, nature and concentration of ligands and complex ions, effectively regulate the bioavailability of the metal ions in water, air and soils (Westrup et al., 2005; Zimmermann et al., 2010; Fiedler et al., 2004; Fritzen et al., 2006; Saenz et al., 2010);

In biological systems, Cd^{2+} is able to stimulate the production of reactive oxygen species (ROS) and reactive nitrogen species, which are responsible, at least in part, for its toxicity (Stohs et al., 2001).

Cd^{2+} was shown to induce its toxic effects under *in vitro* conditions, on developing cortical cells and on immature hippocampal

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doi:10.1016/j.fct.2011.07.006

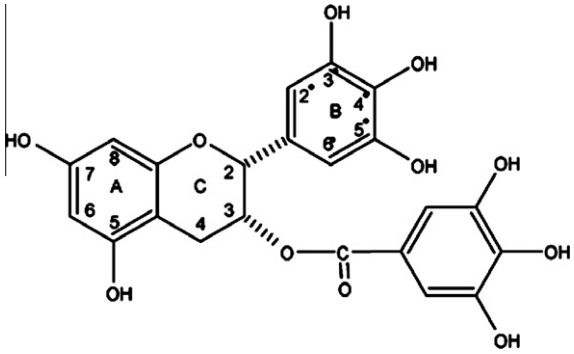

Fig. 1. Chemical Structure of EGCG.

slices (Ohtani-Kanek et al., 2008; Rigon et al., 2008), and *in vivo* on developing brain (Rai et al., 2010; Amara et al., 2011). Although the Cd^{2+} entrance into the adult central nervous system (CNS) is limited, developmental neurotoxicity seems to occur, at least in part, as a result of blood–brain barrier (BBB) immaturity (Choudhuri et al., 1996; Rai et al., 2010; Amara et al., 2011). Moreover, high Cd^{2+} levels are known to impair BBB function (Bar-Sela et al., 2001).

Although Cd^{2+} leads to oxidative stress in the CNS (Sinha et al., 2008; Goncalves et al., 2010) specially during early development (Rai et al., 2010), the complete understanding about molecular mechanisms related to Cd^{2+}-induced neurotoxicity remains unknown.

Apoptosis is a dynamic multistep mechanism of cell death controlled by mitochondria (Bayir and Kagan, 2008) – major site of ROS generation – and is related to the cytotoxicity induced by metals (Pulido and Parrish, 2003; Belyaeva et al., 2008).

CNS is especially susceptible to oxidative stress due to its high oxygen turnover, as well as of its high amount of polyunsaturated fatty acids (Floyd and Hensley, 2002). Therapeutic use of antioxidants from diet appears to prevent a range of aging effects as well as neurodegenerative diseases (Mandel and Youdim, 2004). Several studies have suggested that antioxidant compounds, such as polyphenols, help in the treatment of neurodegenerative diseases (Rajeswari and Sabesan, 2008; Ritz et al., 2008; Wang et al., 2009). However, the beneficial effects of such compounds toward metal-induced neurotoxicity are scarce.

Considering (i) mitochondrial ROS generation an important event related to neurodegenerative/neuropathological conditions, (ii) Cd^{2+}-induced neurotoxicity is related to oxidative stress generation (Chen et al., 2011) and (iii) polyphenol compounds have presented significant beneficial effects in pathological conditions related to oxidative stress, the aim of the present study was to investigate the effect of EGCG against Cd^{2+}-induced mitochondrial damage, using mitochondrial-enriched fractions from rat brain. Biochemical parameters related to the mitochondrial function, as well as oxidative stress related parameters, were evaluated. Moreover, the potential chelating effects of EGCG toward Cd^{2+} were examined in order to elucidate potential mechanisms of mitotoxicity.

2. Material and methods

2.1. Material

Cadmium chloride and $AgNO_3$ were purchase from Merk. EGCG, 2-thiobarbituric acid, 55′-dithiobis-(2-nitrobenzoic-acid) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis MO, USA). All other chemicals were purchased from common commercial suppliers.

2.2. Animals

Male 14-day-old Wistar rats were collected from the Federal University of Santa Catarina (UFSC) breeding colony. Rats were maintained in an air-conditioned room (22–25 °C) on a 12 h light/dark cycle with water and food available *ad libitum*. They were treated, manipulated and killed according to the "Principles of laboratory animal care." (NIH publication No. 80-23, revised 1996) and approved by the local Ethical Committee for Animal Research (CEUA/UFSC PP00345).

2.3. Preparation of mitochondrial enriched fractions

Mitochondrial-enriched fractions were prepared as described previously (Whittaker, 1962), with minor modifications (Franco et al., 2007). Briefly, 14-day-old male Wistar rats were killed by decapitation. The whole brains were removed and homogenized on ice in 10 volumes of isolation medium (10 mM HEPES buffer pH 7.0 containing 220 mM mannitol, 68 mM sucrose, 10 mM KCl and 0.1% serum albumin), and the homogenate was centrifuged at 4 °C for 10 min at 1000 *g*. The supernatant was then centrifuged at 11,500*g* for 10 min at 4 °C, resulting in a myelin-rich supernatant and a pellet (P2) consisting of synaptosomes and free mitochondria. The supernatant was discarded, and the pellet was resuspended in the isolation medium but without albumin. The mitochondrial-enriched fractions were kept on ice for 10–15 min until the experiments were performed. *N* ranges from 5 to 8 independent fractions as indicated in the figure legends and each independent fraction was obtained from 4 brains homogenates.

2.4. Incubations

P2 (approximately 4 mg of protein) was incubated with different cadmium chloride concentrations (100, 200 or 300 μM) and/or different EGCG concentrations (10, 50 or 100 μM) in a medium containing 10 mM HEPES buffer (pH 7.0), 220 mM mannitol, 68 mM sucrose and 10 mM KCl (total incubation volume = 300 μL). Incubations were carried out for 2 h at 25 °C. Epigallocatechin-3-gallate was dissolved in NaCl 0.9%. After incubations, mitochondrial function, thiobarbituric acid reactive substances (TBARS) or GSH content were determined.

2.5. Thiobarbituric acid reactive substances (TBARS) assay

The thiobarbituric acid (TBA) assay was used to assess lipid peroxidation as previously described (Ohkawa et al., 1979). Briefly, samples were incubated in a reaction media containing 0.28% 2-thiobarbituric acid, 1.2% SDS, and 0.45 M/0.12 M acetic acid/HCl buffer (pH 3.4). After incubation at 95 °C for 60 min, TBARS were measured at 532 nm and compared to a standard curve of malondialdehyde.

2.6. Assessment of mitochondrial function

Mitochondrial function was assessed using MTT reduction assay. This assay is based on the ability of the mitochondrial dehydrogenases to metabolize MTT to formazan, a reaction that takes place if the mitochondrial preparation is functionally intact. Briefly, after the preincubation of P2 with Cd^{2+} and/or EGCG (2 h at 25 °C), the reaction medium (300 μL) was incubated with 300 μL of 1.2 mM MTT for 30 min at 25 °C. The purple formazan crystals were pelleted by centrifugation, and the supernatant was discarded. The pellets were dissolved in DMSO and the formazan was quantified spectrophotometrically at 550 nm. Data were expressed as a percentage of control.

2.7. Assessment of glutathione content

Glutathione content was measured as nonprotein thiols (NPSH) according to a method previously described (Ellman, 1959), with minor modifications. Briefly, after the preincubation of P2 with Cd^{2+} and/or EGCG (2 h at 25 °C), 300 μL of trichloroacetic acid 10% was added to the reaction medium (300 μL). After centrifugation (4000*g* at 4 °C for 10 min), the protein pellet was discarded and free thiol groups were determined in the clear supernatant (which was neutralized with 0.1 M NaOH).

2.8. Interaction of Cd^{2+} and EGCG

A standard solution containing 0.0980 ± 0.0013 mol/L Cd^{2+} was prepared by dissolving $CdCl_2 \cdot H_2O$ in 100 mL of water, and the concentration of Cd^{2+} determined by volumetric titration with $AgNO_3$. The ligand was EGCG and a standard solution 1.0×10^{-3} mol/L was prepared in an aqueous solution containing NaCl 0.9% m/v. Calibration solutions were prepared daily by appropriate dilution of the metal stock solutions and all analytical procedures were carried out in a laboratory with the TROX® (class 100) air filtrating system provides control of particles: 0.3–0.5 μm/min. All other inorganic reagents were the best available analytical reagent grade.

UV–vis spectrophotometric measurements were carried out at 24.0 ± 1.0 °C on a FEMTO 800 XI spectrophotometer equipped with deuterium and tungsten lamps and 1 cm quartz cell, calibrated with NIST traceable UV/Vis reference materials.