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ScienceDirect
Free Radical Biology & Medicine 43 (2007) 546–556
www.elsevier.com/locate/freeradbiomed

Original Contribution

A novel approach of proteomics and transcriptomics to study the mechanism of action of the antioxidant–iron chelator green tea polyphenol (-)-epigallocatechin-3-gallate

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Received 20 February 2007; revised 25 April 2007; accepted 7 May 2007
Available online 16 May 2007

Abstract

Previous findings suggest that the antioxidant–iron chelator green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) may have a neurorescue impact in aging and neurodegenerative diseases to retard or even reverse the accelerated rate of neuronal degeneration. The present study sought a deeper elucidation of the molecular neurorescue activity of EGCG in a progressive neurotoxic model of long-term serum deprivation of human SH-SY5Y neuroblastoma cells. In this model, proteomic analysis revealed that EGCG (0.1–1 μM) affected the expression levels of diverse proteins, including proteins related to cytoskeletal components, metabolism, heat shock, and binding. EGCG induced the levels of cytoskeletal proteins, such as beta tubulin IV and tropomyosin 3, playing a role in facilitating cell assembly. In accordance, EGCG increased the levels of the binding protein 14-3-3 gamma, involved in cytoskeletal regulation and signal transduction pathways in neurons. Additionally, EGCG decreased protein levels and mRNA expression of the beta subunit of the enzyme prolyl 4-hydroxylase, which belongs to a family of iron–oxygen sensors of hypoxia-inducible factor (HIF) prolyl hydroxylases that negatively regulate the stability and degradation of several proteins involved in cell survival and differentiation. Accordingly, EGCG decreased protein levels of two molecular chaperones that were associated with HIF regulation, the immunoglobulin-heavy-chain binding protein and the heat shock protein 90 beta. Thus, the present study sheds some light on the antioxidative–iron chelating activities of EGCG underlying its neuroprotective/neurorescue mechanism of action, further suggesting a potential neurodegenerative-modifying effect for EGCG.

Keywords: EGCG; Proteomics; Cytoskeletal proteins; 14-3-3 Gamma; Prolyl 4-hydroxylase beta; BiP; HSP90 beta

Introduction

Oxidative damage of neuronal biomolecules and increased accumulation of iron in specific brain areas are considered major pathological aspects of neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) [1]. In line with this evidence, it is not surprising that neuroprotective therapies were aimed to interfere with the cytotoxic oxidative stress (OS) process and particular attention has been placed on the study of the neuroprotective actions of the antioxidant and iron chelator compounds tea flavonoids and especially the major green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) [2–5]. Indeed, the importance of polyphenolic flavonoids in enhancing cell resistance to OS goes beyond the simple scavenging activity and is mostly interesting in those pathologies where OS plays an

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; BiP, immunoglobulin-heavy-chain binding protein HSP 70 kDa protein 5; CID, collision-induced dissociation; 2D, two-dimensional; DEPC, diethylpyrocarbonate; DTT, dethiothrectol; EGCG, epigallocatechin-3-gallate; ERK, extracellular signal-regulated kinase; HNR G, heterogeneous nuclear ribonucleoprotein G; HIF, hypoxia-inducible factor; HSP, heat shock protein; IEF, isoelectric focusing; IPG, immobilized pH gradient; MAPK, mitogen-activated protein kinases; MS, mass spectrometry; OS, oxidative stress; PBS, phosphate-buffered saline; PC12, rat pheochromocytoma; PD, Parkinson's disease; PCR, polymerase chain reaction; **p*I*s**, isoelectric points; PKC, protein kinase C; RT, reverse-transcribed; TBS, Tris-buffered saline; TfR, transferrin receptor; UBE2R2, ubiquitin-conjugating enzyme E2R 2.

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doi:10.1016/j.freeradbiomed.2007.05.011

important role. Numerous studies in the past 10 years have shown that polyphenols have in vitro and in vivo activities in preventing and/or reducing the deleterious effects of oxygen-derived free radicals associated with various chronic diseases [6]. Thus, evaluation of the neuroprotective effects of acute and chronic administration of EGCG in various cellular and animal models of PD and AD revealed various novel molecular targets including the extracellular mitogen-activated protein kinases (MAPK), protein kinase C (PKC), antioxidant enzymes, and survival genes and proteins associated with mitochondrial function, such as Bcl-2 family members [7–13]. In addition, EGCG affected the processing of the amyloid precursor protein (APP) pathway and regulated transferrin receptor (TfR) in human SH-SY5Y neuroblastoma cells [14]. Recently, we demonstrated that EGCG (0.1 and 1 μM) improved cell morphology and promoted neurite outgrowth in a progressive model of cell death induced by long-term serum deprivation of rat pheochromocytoma (PC12) cell cultures [13,14].

Proteome maps of rat brains treated with grape seed extract [15] and of human vascular senescent endothelial cells treated with EGCG [16] demonstrated altered expression of various proteins, which were associated with cell cycle and cytoskeleton and may mediate cell viability. Indeed, the possibility of high-throughout screening using proteomic techniques should provide more comprehensive overview of the interaction of proteins, the interplay among processes, and the context in which a specific molecule or pathway may be operating in the mechanism of action of EGCG.

The present study aimed to gain insight into the molecular mechanism of the neurorescue effect of EGCG, in a progressive neurorescue model of human SH-SY5Y neuroblastoma cells, by employing proteomic analysis combined with transcriptomic tools. Here, we report that EGCG affects the expression levels of diverse proteins, including those related to cytoskeletal components, metabolism, binding proteins, and heat shock proteins (HSPs), associated with its multimodal neuroprotective/neurorescue effects described by us recently: induction of neurite outgrowth [13], degradation of the cell death regulator Bad [8], and iron chelation [14].

Material and methods

Cell culture

Human SH-SY5Y neuroblastoma cells were plated in 100-mm culture dishes and cultured in Dulbecco's modified Eagle's medium (4500 mg/L glucose) containing 10% fetal calf serum and a mixture of 1% penicillin/streptomycin/nystatin. Cell cultures were incubated at 37 °C in a humid 5% CO_2–95% air environment. When cells reached the required confluence, they were seeded (2×10^6) onto 100-mm plates in culture medium supplemented with full serum for 1 day at 37 °C. Continuously, the medium was replaced into serum-deprived medium for 3 days and, further on, the cells were incubated with or without various concentrations of EGCG (0.1 and 1 μM) in fresh serum-free medium for an additional 2 days at 37 °C. Cells incubated with or without full-serum medium were used as controls.

Protein and total RNA isolation protocols

Isolation of protein and total RNA was performed, using Tri Reagent isolation reagent according to the manufacture's protocol (Sigma Chemical Co., St. Louis, MO, USA). The TriReagent suspensions were mixed thoroughly with chloroform and centrifuged (12,000*g*, 15 min, 4 °C). The organic phase (containing proteins) and the interphase (containing DNA) were further treated with 100% ethanol and centrifuged (10,000*g*, 15 min, 4 °C). The protein was precipitated with isopropanol and centrifuged (12,000*g*, 15 min, 4 °C). The protein pellet was washed several times with 0.3 M guanidine hydrochloride in 95% ethanol according to the manufacture's protocol (Sigma). Protein content was determined using the Bradford method. The aqueous phase containing the RNA was precipitated with isopropanol and centrifuged (12,000*g*, 15 min, 4 °C). The RNA pellet was washed twice with 70% ethyl alcohol (7500*g*, 10 min), followed by one wash with 96% ethyl alcohol (12,000*g*, 10 min), resuspended in diethylpyrocarbonate (DEPC)-treated water, and incubated for 5 min at 56 °C to facilitate resuspension. Total RNA aqueous solution was treated with DNase–Rnase-free (Roche Diagnostics, Mannheim, Germany) for 30 min at 37 °C and subsequently extracted by a round of phenol:chloroform: isoamylalcohol (25:24:1) followed by one round of chloroform:isoamylalcohol (24:1). After precipitation with sodium acetate (0.3 M) and isopropanol, the RNA pellet was washed as described above with 70 and 96% ethyl alcohol and resuspended in DEPC-treated water. RNA sample concentrations were determined by UV spectrophotometer at 260 nm. The quality of RNA was assessed by the absorbance at 260/280 nm ratio and by agarose gel analysis through direct visualization of 18S and 28S rRNA bands.

Two-dimensional (2D) gel electrophoresis and image analysis

Two-dimensional polyacrylamide gel electrophoresis (PAGE) was performed under conditions essentially as described by the manufacture's protocol (Bio-Rad, Hercules, CA, USA) using 110-mm pH 3–10 immobilized pH gradient (IPG) strips and Criterion (4–20%) gels. Protein samples were dissolved in buffer containing 7 M urea, 2 M thiourea, 65 mM dithiothreitol (DTT), 0.125% (v/v) Biolytes 3-10, 2% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid, and bromophenol blue. For the first-dimension separation, 200 μg of protein was applied to a dehydrated IPG strip and isoelectric focusing (IEF) was carried out at room temperature as follows: passive dehydration for 1 h and then at linear gradient from 50 to 8000 V for 12 h. Subsequently, strips were placed on Criterion gels and the second-dimension separation was carried out at 200 V for 75 min. Following electrophoresis, gels were visualized by SeeBend Forte protein staining solution following the manufacturer's recommendation (Bio-Rad). The gel images were acquired with a Bio-Rad Fluor-S MultiImager and spots were indexed using the PDQuest 2-D software for comparisons and quantitation of 2D gel spots.