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Green Tea Epigallocatechin 3-Gallate Accumulates in Mitochondria and Displays a Selective Antiapoptotic Effect Against Inducers of Mitochondrial Oxidative Stress in Neurons

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Abstract

Epigallocatechin-3-gallate (EGCG) is a major flavonoid component of green tea that displays antiapoptotic effects in numerous models of neurotoxicity. Although the intrinsic free radical scavenging activity of EGCG likely contributes to its antiapoptotic effect, other modes of action have also been suggested. We systematically analyzed the antiapoptotic action of EGCG in primary cultures of rat cerebellar granule neurons (CGNs). The dose-dependent protective effects of EGCG were determined after coincubation with eight different stimuli that each induced neuronal apoptosis by distinct mechanisms. Under these conditions, EGCG provided significant neuroprotection only from insults that induce apoptosis by causing mitochondrial oxidative stress. Despite this selective antiapoptotic effect, EGCG did not significantly alter the endogenous activities or expression of Mn^{2+}-superoxide dismutase, glutathione peroxidase, Nrf2, or Bcl-2. Subfractionation of CGNs after incubation with ^{3}H-EGCG revealed that a striking 90–95% of the polyphenol accumulated in the mitochondrial fraction. These data demonstrate that EGCG selectively protects neurons from apoptosis induced by mitochondrial oxidative stress. This effect is likely due to accumulation of EGCG in the mitochondria, where it acts locally as a free radical scavenger. These properties of EGCG make it an interesting therapeutic candidate for neurodegenerative diseases involving neuronal apoptosis triggered by mitochondrial oxidative stress. *Antioxid. Redox Signal.* 11, 469–480.

Introduction

EPIGALLOCATECHIN 3-GALLATE (EGCG) is a major polyphenolic constituent of green tea that has been shown to protect neurons from toxic insults in diverse *in vitro* paradigms. For instance, EGCG mitigates oxidative stress and reduces apoptosis induced by hydrogen peroxide in a variety of neuronal cell types, including motoneurons (22), N18D3 mouse neuroblastoma x dorsal root ganglion hybrid cells (21), spiral ganglion cells (50), and RGC-5 retinal ganglion cells (52). EGCG protects SH-SY5Y human neuroblastoma cells from serum deprivation, 3-hydroxykynurenine, or 6-hydroxydopamine toxicity (2, 16, 27). Similarly, EGCG rescues rat PC12 cells from serum withdrawal, lead toxicity, or paraquat-induced apoptosis (6, 15, 36). EGCG also reduces apoptosis caused by exposure of fetal rhombencephalic neurons to ethanol (1) and protects hippocampal neurons from β-amyloid toxicity (7). Finally, EGCG rescues primary dopamine neurons from 1-methyl-4-phenylpyridinium (MPP+) toxicity (44) and attenuates nitric oxide–induced death of SN4741 dopaminergic cells (26).

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In addition to the neuroprotective effects of EGCG observed *in vitro*, this catechin also preserves neuronal survival and function in several *in vivo* models of neurodegeneration. For example, oral administration of EGCG protects mice from the dopaminergic toxicity caused by the Parkinson neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). EGCG treatment prevents the MPTP-induced loss of dopamine neurons from the substantia nigra pars compacta and preserves striatal dopamine levels in mice (28). In a similar manner, EGCG is protective in a mouse model of familial amyotrophic lateral sclerosis (ALS). Oral dosing of EGCG to transgenic mice expressing a human G93A mutant SOD1 (Cu, Zn-superoxide dismutase) gene significantly delays symptom onset and moderately extends life span when compared with those of vehicle-treated mice (23, 51). EGCG also reduces photoreceptor degeneration and improves motor function in a *Drosophila* model of Huntington disease (10). Finally, oral administration of EGCG to Swedish mutant amyloid precursor protein (APPsw)-overexpressing transgenic mice substantially decreases amyloid plaque burden and reduces cognitive impairment (42). Collectively, these findings indicate that EGCG may be a viable therapeutic candidate for neurodegenerative diseases such as Alzheimer's or Parkinson's (11, 47).

Although many studies support the hypothesis that the neuroprotective effects of EGCG are directly related to its intrinsic antioxidant properties, including the scavenging of reactive oxygen and nitrogen species and the chelation of transition metals such as iron and copper, many other modes of action for this catechin have been suggested (reviewed in 14, 35, 37). For instance, EGCG has been shown to stimulate prosurvival phosphatidylinositol 3-kinase (PI3K)/AKT and protein kinase C (PKC) pathways (21, 22, 27). Conversely, EGCG appears to inhibit proapoptotic kinase pathways including the stress-activated protein kinases [c-Jun-NH_2 terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase] and Janus kinases (JAKs) (12, 18, 20). Alternatively, EGCG has been shown under some conditions to upregulate the expression of antiapoptotic proteins like Bcl-2 while concomitantly downregulating proapoptotic molecules such as Bax and Bad (2, 27, 48). In addition, EGCG can act as an inducer of the Nrf2 (nuclear factor erythroid-derived 2–related factor 2) transcription factor, which results in the induction of various antioxidant genes including Mn^{2+}-superoxide dismutase (MnSOD) and glutathione peroxidase (GSHpx) (29, 39, 40). Last, EGCG has been shown to inhibit glutamate-induced Ca^{2+} influx and excitotoxicity in PC12 cells (25). Given the diversity of cellular systems and animal models that have been used to investigate the neuroprotective mechanism of EGCG, it is presently unclear how each of these multifunctional activities may contribute to its ability to rescue neurons from various toxic insults.

In the present study, we took a systematic approach to analyze the neuroprotective efficacy of EGCG against a diverse array of toxic insults that each induced apoptosis in primary cultures of rat cerebellar granule neurons (CGNs) through distinct mechanisms. These cultures are highly homogeneous, with ~95% of the cells being CGNs (9). In addition, this cell model has been used extensively to examine molecular mechanisms involved in neuronal apoptosis (8, 9, 45). Interestingly, we found that EGCG selectively protects CGNs from apoptosis induced by mitochondrial oxidative stress. Furthermore, our data suggest that this selective neuroprotective effect is likely due to a dramatic and previously unreported accumulation of EGCG in neuronal mitochondria.

Materials and Methods

Reagents

MG-132, brefeldin A, EGCG, and HA14-1 were obtained from Calbiochem (San Diego, CA). *Clostridium difficile* toxin B was a kind gift from Dr. Klaus Aktories and Dr. Torsten Giesemann (Albert-Ludwigs-Universität Freiburg, Germany). L-Glutamic acid, *tert*-butylhydroperoxide, and SIN-1 were obtained from MP Biomedicals (Solon, OH). Polyclonal (N-19) BCL-2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Hoechst dye 33258, 4,6-diamidino-2-phenylindole (DAPI), monoclonal antibody against É-tubulin, and the GSHpx assay kit were obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies to OPA1 and MnSOD were purchased from BD Biosciences (San Jose, CA). A polyclonal Nrf2 antibody was obtained from Abcam (Cambridge, MA). The MTT viability assay kit and the mitochondria/cytosol fractionation kit were from BioArray Systems (Hayward, CA) and Alexis/Axxora (San Diego, CA), respectively. The assay kit for SOD activity was from Oxis International, Inc. (Foster City, CA). Polyclonal antibody against β-actin was obtained from Cell Signaling (Beverly, MA). Horseradish peroxidase–linked secondary antibodies and reagents for enhanced chemiluminescence detection were from Amersham Biosciences (Piscataway, NJ). Polyclonal antibody to active caspase-3 was from Promega (Madison, WI). Cy3- and FITC-conjugated secondary antibodies for immunofluorescence were from Jackson Immunoresearch Laboratories (West Grove, PA). ^{3}H-EGCG (20 μCi total; 10 Ci/mmol) was custom synthesized by American Radiolabeled Chemicals, Inc. (St. Louis, MO). MG132, brefeldin A, and HA14-1 each was solubilized as a stock solution in DMSO. Stock solutions of glutamate/glycine, *tert*-butylhydroperoxide, and SIN-1 were made up in sterile water.

CGN culture

CGNs were isolated from postnatal day 7 Sprague–Dawley rat pups of both sexes (15–19 g), as previously described (32). CGNs were plated on 35-mm diameter plastic dishes coated with poly-L-lysine at 4.0×10^6 cells/well in basal modified Eagle's medium containing 10% fetal bovine serum, 25 m*M* KCl, 2 mM L-glutamine, and penicillin/streptomycin (100 U/ml/100 μg/ml) (Life Technologies, Inc., Gaithersburg, MD). Cytosine arabinoside (10 μM) was added to the culture medium 24 h after plating to limit the growth of nonneuronal cells. With this protocol, cultures were ~95% pure for granule neurons. Typically, experiments were performed after 6–7 days in culture.

CGN apoptosis and MTT viability assays

For quantification of CGN apoptosis, cells were exposed to various proapoptotic stimuli ± EGCG (5, 10, or 20 μM) for 24 h. After incubation, CGNs were fixed in 4% paraformaldehyde, and nuclei were stained with Hoechst dye. CGNs containing condensed or fragmented chromatin or both were

scored as apoptotic. In general, ~500 CGNs were quantified for apoptosis from each 35-mm well by randomly counting ~10 60× fields. Apoptosis counts were performed inblinded fashion, and each treatment was performed in duplicate. Graphic data of apoptosis counts represent the mean ± SEM for the number of independent experiments performed (with each experiment performed on duplicate wells per condition). For the MTT viability assay, CGNs were incubated in either control medium alone or containing HA14-1 (15 μM) ± EGCG (5, 10, or 20 μM). After 16 h of incubation, 187.5 μl of CellQuanti-MTT assay reagent (15 μl per 80-μl culture medium) was added directly to each well. After an additional 4 h of incubation at 37°C/10% CO_2, solubilization solution (1,250 μl/well) was added directly to each well. Culture plates were then mixed on an orbital shaker for 1 h at 25°C. After solubilization, the entire volume of each well was transferred to individual 15-ml conical tubes and centrifuged for 2 min at 1,300 rpm. A 500-μl aliquot was drawn from each sample, and the absorbance was read at 570 nm (minus absorbance at 650 nm). Data averages were calculated relative to the control mean absorbance for each independent experiment.

Subcellular fractionation

Adhered CGNs were washed once with 2 ml of phosphate-buffered saline (PBS; pH 7.4) and were then incubated for 20 min on ice with 200 μl cytosolic extraction buffer. Duplicate samples were scraped from the wells and combined into 1.5-ml microfuge tubes. Samples were homogenized with 40 strokes with a dounce homogenizer. The samples were centrifuged at 720 *g* for 10 min at 4°C, and the supernatants were transferred to new 1.5-ml microfuge tubes for mitochondrial fractionation. Mitochondria were isolated by centrifugation of the supernatants at 10,000 *g* for 30 min at 4°C. After centrifugation, the supernatants were transferred to different microfuge tubes and labeled "cytosol"; the mitochondrial pellets were resuspended in either 110 μl of mitochondrial extraction buffer for immunoblotting or 50 μl of mitochondrial extraction buffer for the GSHpx assay.

Cell lysis and immunoblotting

After treatment, whole-cell lysates of CGNs for Western blotting were prepared essentially as previously described (34). Equivalent amounts of cell protein (determined by using a commercially available protein assay kit; BCA, Pierce Chemical Co., Rockford, IL) were electrophoresed through polyacrylamide gels, and resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ). For immunoblotting, PVDF membranes were blocked in PBS (pH 7.4) with 0.1% Tween 20 (PBS-T) containing 1% BSA and 0.01% sodium azide for 1 h at 25°C. The primary antibody was diluted in blocking solution and added to the membranes for 1 h. The membranes were then washed for 25 min with PBS-T changing the wash in 5-min intervals. Membranes were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies diluted in PBS-T. Secondary antibody solution was then washed off over a 25-min period with PBS-T in 5-min wash-change intervals. This was followed by detection of immunoreactive proteins with enhanced chemiluminescence. Blots shown are representative of a minimum of three independent experiments.

Immunocytochemistry

After treatment, CGNs were fixed in 4% paraformaldehyde, washed once in PBS, and then permeabilized and blocked in 0.2% Triton X-100 and 5% BSA in PBS (pH 7.4). Primary antibodies were diluted in 2% BSA and 0.2% Triton X-100 in PBS. Cells were incubated in the primary antibody for approximately 16 h at 4°C. They were subsequently washed 5 times in PBS over a 30-min period and then incubated for 1 h at 25°C with Cy3- or FITC-conjugated secondary antibodies and DAPI diluted in 2% BSA and 0.2% Triton X-100 in PBS. The cells were washed 5 more times over a 30-min period with PBS before the addition of anti-quench composed of 0.1% *p*-phenylenediamine in 75% glycerol in PBS. Fluorescent images were captured by using a 63x oil immersion objective on a Zeiss Axioplan 2 microscope equipped with a Cooke Sensicam deep-cooled charge-coupled device (CCD) camera and a Slidebook software-analysis program for digital deconvolution (Intelligent Imaging Innovations, Inc., Denver, CO).

SOD activity assay

CGNs were analyzed with a spectrophotometric assay to measure SOD activity. One day before the assay, CGNs were incubated in control medium ± EGCG (20 μM). After 24 h, cells were lysed as described earlier, and the SOD assay was performed essentially as described by the manufacturer (Oxis International, Inc.). Each sample contained 750 μl of assay buffer, 190 μl of CGN lysate, 30 μl of reagent 2 (R2), and 30 μl of reagent 1 (R1). R2 was added first, and the absorbance at 525 nm was measured as the t = 0 baseline. R1 was subsequently added, and the samples were incubated at 37°C for 15 min while the change in absorbance was measured. The relative amount of SOD activity in cell lysates was measured as an increase in absorbance at 525 nm. Data shown are the means ± SEM of three independent experiments, each performed in triplicate.

GSHpx activity assay

One day before the assay, CGNs were incubated in control medium ± EGCG (20 μM). After 24 h, CGNs were fractionated according to the *"Subcellular fractionation"* procedure described earlier. Mitochondrial fractions were analyzed for GSHpx activity, essentially as described by the manufacturer (Sigma/Aldrich, St. Louis, MO). In brief, each sample contained 890 μl of assay buffer, 50 μl of mitochondrial fraction, 50 μl NADPH, and *tert*-butyl hydroperoxide solution (final concentration of 300 μM). After reagent addition and mixing by inversion, the absorbance at 340 nm was measured over a 14-min period of incubation at 37°C. The relative amount of GSHpx activity in mitochondrial fractions was measured as a decrease in absorbance at 340 nm. Data presented are the means ±SEM of three independent experiments, each performed in triplicate. The purity of the mitochondrial fractions was verified by immunoblotting for OPA1, a large GTPase localized to the inner membrane and intermembrane space of mitochondria (13).

Uptake and distribution of ^{3}H-EGCG in CGNs

CGNs were incubated for 24 h in control medium containing 1 μCi of ^{3}H-EGCG (10 Ci/mmol) in either the absence or presence of 20 μM unlabeled ("cold") EGCG. After incubation, CGNs were lysed, and cytosolic and mitochondrial fractions were obtained by using the procedure described earlier under *"Subcellular fractionation."* Aliquots of cytosolic (100 μl) and mitochondrial (10 μl) fractions were counted with liquid scintillation by using the ^{3}H window. Data for CGNs incubated *without* cold EGCG are presented as a percentage of the total cellular ^{3}H-EGCG that partitioned into the mitochondrial fraction. Results for CGNs incubated *with* cold EGCG are presented as a percentage of the mitochondrial ^{3}H-EGCG observed in CGNs incubated *without* cold EGCG. The data shown are the means ±SEM of triplicate wells from two independent experiments that each produced similar results. The purity of the mitochondrial fractions was verified by immunoblotting for OPA1.

Data analysis and statistics

Graphic data represent the means ±SEM for the number of independent experiments performed. Statistical differences were analyzed by one-way analysis of variance with a *post hoc* Tukey's test. A p value of <0.05 was considered statistically significant. Images, enzyme activity assay data, and immunoblots shown are each representative of at least three independent experiments.

Results

EGCG does not protect CGNs from multiple apoptotic insults that are essentially independent of oxidative stress

In preliminary experiments, we found that EGCG concentrations of 25 μM or higher induced substantial toxicity in cultured rat CGNs after a 24-h incubation period (data not shown). Therefore, in all subsequent experiments, we used EGCG at final concentrations of 5, 10, or 20 μM to assess its neuroprotective efficacy against diverse proapoptotic stimuli. Initially, we examined the effects of EGCG on insults that induce CGN apoptosis by mechanisms that are largely *independent* of oxidative stress. Primary cultures of differentiated CGNs require serum-derived growth factors and depolarizing extracellular potassium (to mimic activity-dependent Ca^{2+} influx) for their survival *in vitro* (9). On removal of serum and depolarizing potassium (*i.e.*, trophic factor withdrawal or *5K* conditions), CGNs die by a mitochondrial apoptosis cascade involving the proapoptotic Bcl-2 family members, Bax and Bim, and activation of caspases -9 and -3 (31, 33, 41). Incubation of CGNs in 5K medium for 24 h induced ~70% apoptosis, and coincubation with EGCG failed to offer any significant protection (Fig. 1A). In addition, we previously showed that the proteasome inhibitor, MG132, induces CGN apoptosis through an enhanced phosphorylation of c-Jun and consequent induction of Bim and activation of caspase-3 (4). Incubation of CGNs with MG132 for 24 h caused ~55% apoptosis, and EGCG again provided no discernible protection (Fig. 1B).

Next, we evaluated the effects of EGCG on CGN apoptosis induced by the Rho family GTPase inhibitor, *C. difficile* toxin B (ToxB). ToxB is a monoglucosyltransferase that

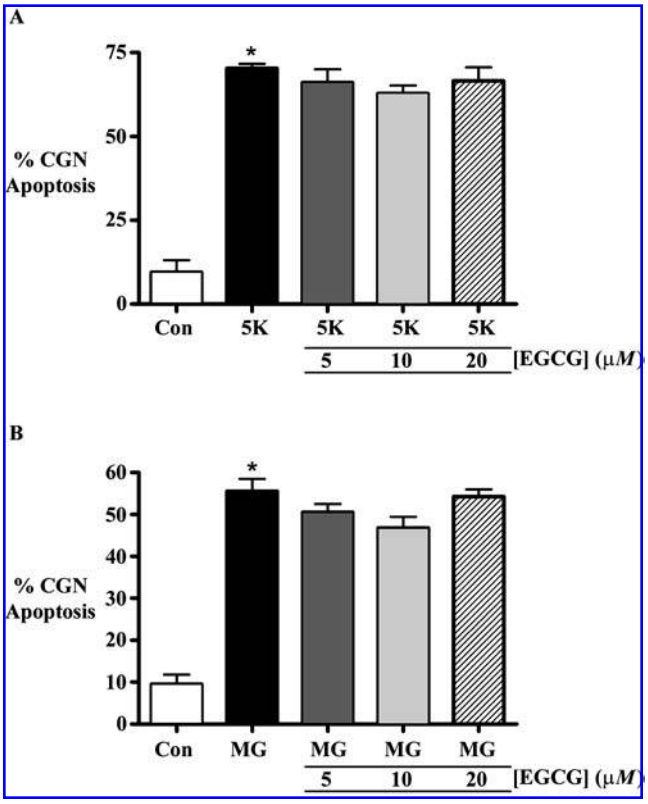


FIG. 1. EGCG does not protect CGNs from either trophic factor withdrawal (5K) or proteasome inhibition. CGNs were incubated in either control medium containing serum and 25 mM potassium chloride (Con), trophic factor withdrawal medium lacking serum and containing only 5 mM potassium chloride (5K; **A**), or control medium containing the proteasome inhibitor, MG132 (MG, 10 μM; **B**). In addition, some cultures were co-incubated with increasing concentrations of EGCG (5, 10, or 20 μM). After incubation for 24 h, CGNs were fixed, and nuclei were stained with Hoechst dye. CGNs displaying condensed or fragmented chromatin or both were scored as apoptotic. *Significantly different from Con ($p < 0.01$).

specifically glucosylates and inactivates Rho, Rac, and Cdc42 GTPases (17). We previously showed that ToxB induces c-Jun/Bim-dependent mitochondrial apoptosis of CGNs, including caspase-9 and -3 activation, by suppressing Rac activity, which is essential for CGN survival (24, 32). Incubation of CGNs with ToxB for 24 h induced ~45% apoptosis, which was unaffected by coincubation with EGCG (Fig. 2A, B).

Finally, we examined the potential of EGCG to mitigate CGN apoptosis induced by the dysregulation of Ca^{2+} homeostasis. First, we used brefeldin A, which causes a blockage of endoplasmic reticulum–to-Golgi transport and consequently elicits inositol 1,4,5-trisphosphate (IP3) receptor–dependent Ca^{2+} toxicity and caspase-9–mediated apoptosis in CGNs (3). Brefeldin A induced approximately 60% apoptosis in CGNs after 24 h, and this toxic effect was slightly (though not significantly) enhanced by EGCG (Fig. 3A). Second, we used glutamate/glycine co-treatment to trigger Ca^{2+}-dependent excitotoxicity in CGNs (49). Incubation of CGNs with glutamate for 24 h induced ~80% apoptosis,

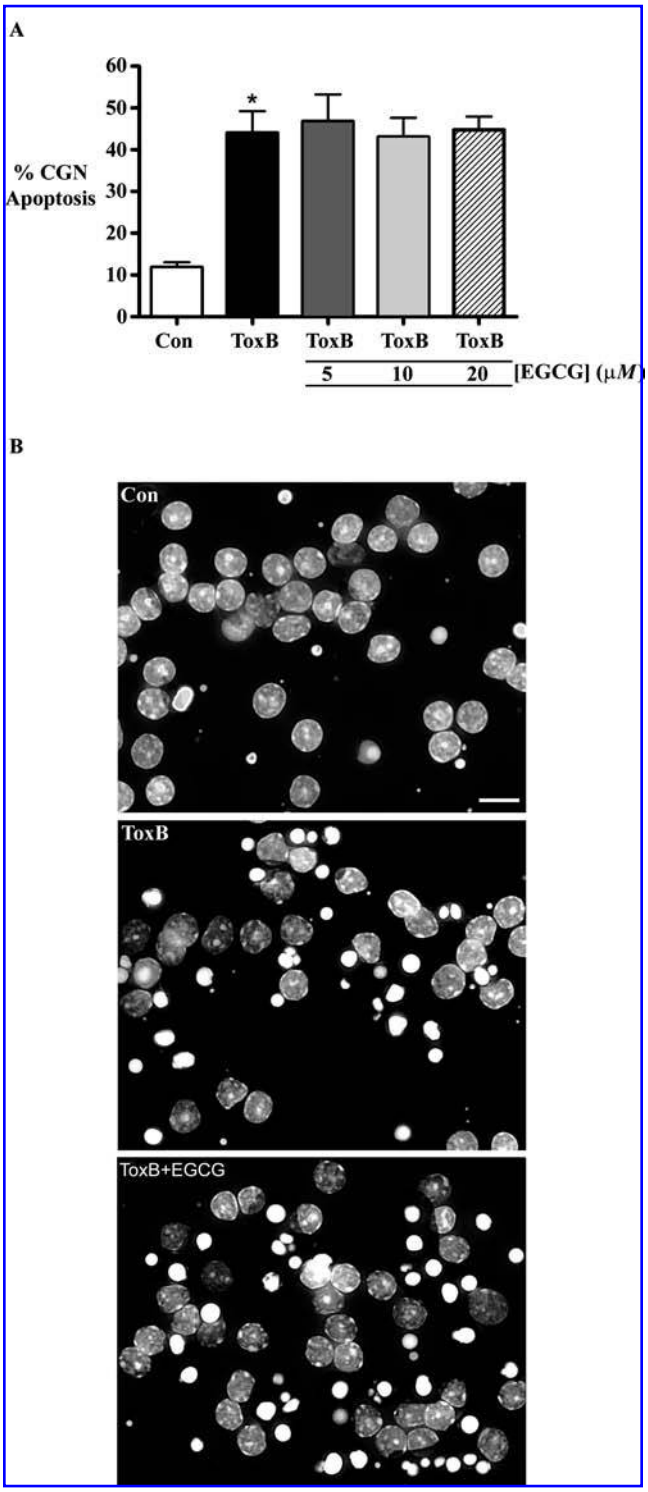


FIG. 2. EGCG does not protect CGNs from the Rho family GTPase inhibitor, ToxB. CGNs were incubated in control medium containing either serum and 25 m*M* potassium chloride (Con) alone or containing *C. difficile* toxin B (ToxB; 50 ng/ml) ± EGCG (5, 10, or 20 μM). After 24 h of incubation, CGNs were fixed and nuclei stained with Hoechst. CGNs displaying condensed or fragmented nuclei or both were counted as apoptotic. Quantification of five independent experiments (in duplicate) is shown in (**A**). *Significantly different from Con ($p < 0.01$). Representative images of Hoechst-stained nuclei are shown in (**B**). Scale bar = 10 μm.

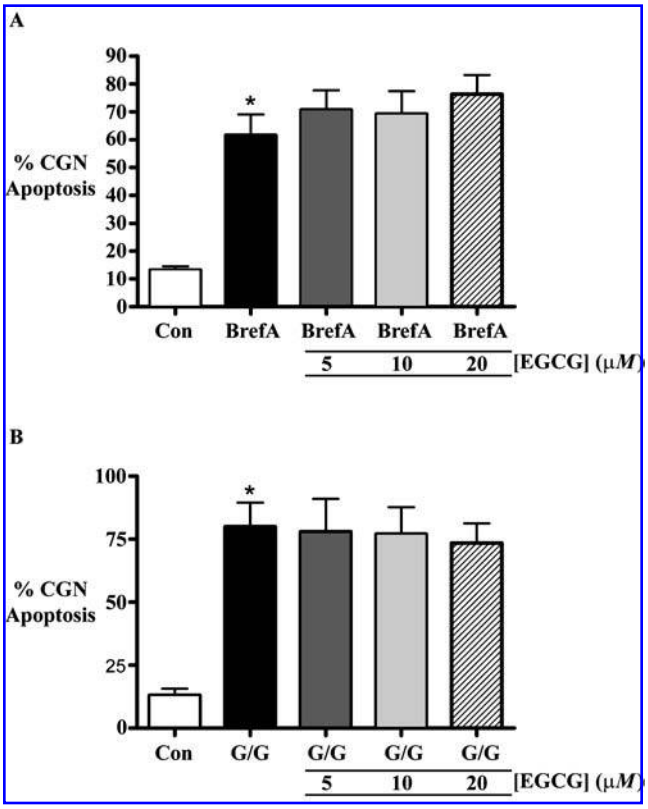
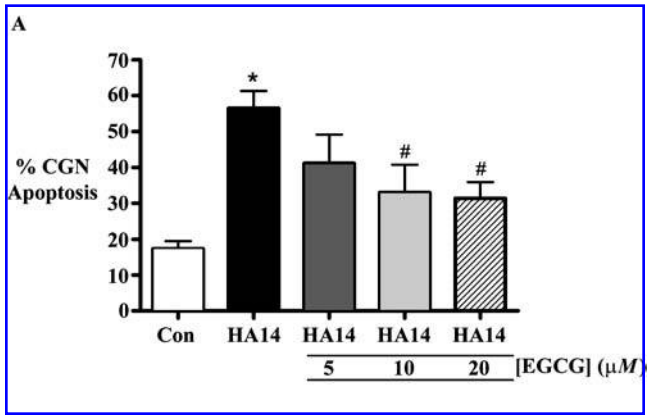


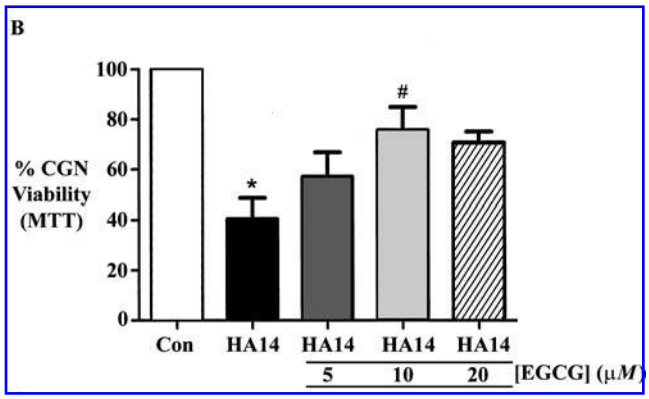
FIG. 3. Endoplasmic reticulum stress and calcium-mediated excitotoxicity in CGNs are not attenuated by co-incubation with EGCG. CGNs were incubated in either control medium containing 25 m*M* potassium chloride and serum (Con), Con medium lacking serum with the endoplasmic reticulum stressor brefeldin A (BrefA, 20 μM; **A**), or in Con medium containing glutamate/glycine (G/G, 100 μM/10 μM; **B**). Some cultures were co-incubated with increasing concentrations of EGCG (5, 10, or 20 μM). G/G was removed after an initial 1-h incubation, and then Con medium lacking serum ± EGCG was re-added to the wells. After 24 h of incubation, cells were fixed and their nuclei stained with Hoechst. CGNs exhibiting condensed or fragmented nuclei or both were considered apoptotic. Data shown represent quantification of at least four independent experiments performed in duplicate. *Significantly different from Con ($p < 0.01$).

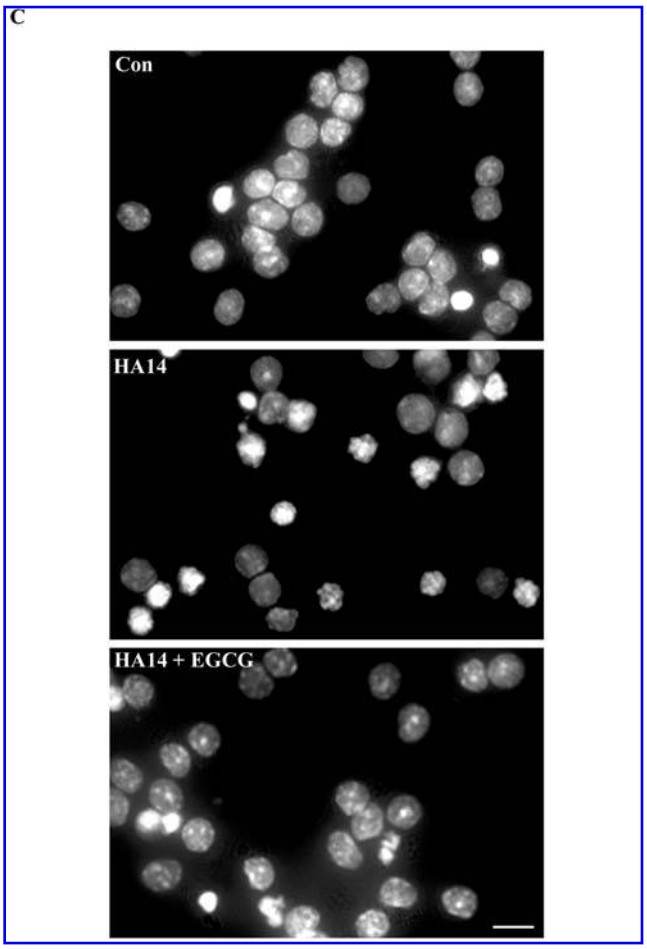
which was essentially unaffected by co-incubation with EGCG (Fig. 3B). Collectively, these data demonstrate that EGCG fails to protect CGNs from a variety of proapoptotic stressors that do not overtly cause oxidative stress.

EGCG significantly protects CGNs from apoptosis induced by mitochondrial oxidative stressors

Next, we analyzed the neuroprotective effects of EGCG against several agents that induce apoptosis principally by triggering mitochondrial oxidative stress. HA14-1 is a small organic compound that mimics the Bcl-2 homology-3 (BH3) domain of proapoptotic family members (*e.g.*, Bim) and consequently inhibits prosurvival Bcl-2 function by binding in a hydrophobic surface groove (*i.e.*, the BH3 domain binding pocket) of this protein (46). We have recently shown that HA14-1 specifically causes mitochondrial oxidative stress







and GSH-sensitive apoptosis in CGNs, including caspase-9 and -3 activation, because of disruption of a novel Bcl-2/GSH interaction and a resultant depletion of the mitochondrial GSH pool (55, 56). Incubation of CGNs with HA14-1 resulted in ~60% apoptosis, as measured by nuclear morphology (Fig. 4A). This result was in agreement with an ~60% decrease in CGN viability measured with an MTT assay (Fig. 4B). Co-incubation with EGCG at concentrations of either 10 or 20 μM significantly inhibited CGN apoptosis induced by the Bcl-2 inhibitor and provided a corresponding increase in cell viability (Fig. 4A–C). It should be noted that the MTT assay results were not entirely consistent with the apoptosis-quantification data in that the 20 μM EGCG concentration did not *significantly* protect CGNs from the HA14-1–induced decrease in viability measured with the MTT assay (Fig. 4B). Nonetheless, a definite trend was seen toward greater viability with this dose of EGCG, although it did not quite reach statistical significance.

In addition to HA14-1, we examined the effects of EGCG on CGN apoptosis caused by either *tert*-butylhydroperoxide (tBH) or SIN-1 (a generator of peroxynitrite). Previous studies using N2A and SH-SY5Y neuroblastoma cell lines showed that mitochondrial depolarization and apoptosis induced by tBH are significantly attenuated by an antioxidant peptide that selectively accumulates in mitochondria (53). Moreover, SIN-1 toxicity in astrocytes is markedly enhanced by selective depletion of the mitochondrial GSH pool (38). These prior findings indicate that both tBH and SIN-1 induce apoptosis largely by triggering mitochondrial oxidative stress. Incubation of CGNs with either tBH or SIN-1 for 24 h induced ~45% or 75% apoptosis, respectively (Fig. 5A and B). Co-incubation with EGCG at concentrations of 10 or 20 μM significantly inhibited CGN apoptosis induced by each of these mitochondrial oxidative stressors. Furthermore, SIN-1 caused a marked disruption of the microtubule network in CGNs, which was largely prevented by EGCG (Fig. 6). Thus, EGCG demonstrates a significant neuroprotective action against agents known to cause apoptosis through induction of mitochondrial oxidative stress.

FIG. 4. CGNs are significantly protected by EGCG from apoptosis induced by the Bcl-2 inhibitor HA14-1. CGNs were incubated with either control medium containing serum and 25 mM potassium chloride (Con) or medium lacking serum and containing the Bcl-2 inhibitor, HA14-1 (HA14, 15 μM). Some cultures were also co-treated with increasing concentrations of EGCG (5, 10, or 20 μM). After 24-h incubation, cells were either fixed and their nuclei stained with Hoechst (**A**) or incubated with MTT assay reagents to assess viability (**B**). Hoechst-stained CGNs displaying condensed or fragmented nuclei were considered apoptotic. Additionally, percentage cell viability relative to Con was measured by the reduction of MTT, a tetrazolium salt. The Con was set as the standard at 100% cell viability. Data represent the mean ± SEM ($n = 7$). *Significantly different from Con ($p < 0.01$); #significantly different from HA14 alone; $p < 0.01$ *vs.* HA14 alone (**A**) and $p < 0.05$ *vs.* HA14 alone (**B**). Representative images of Hoechst-stained nuclei are shown in (**C**). Scale bar = 10 μm.

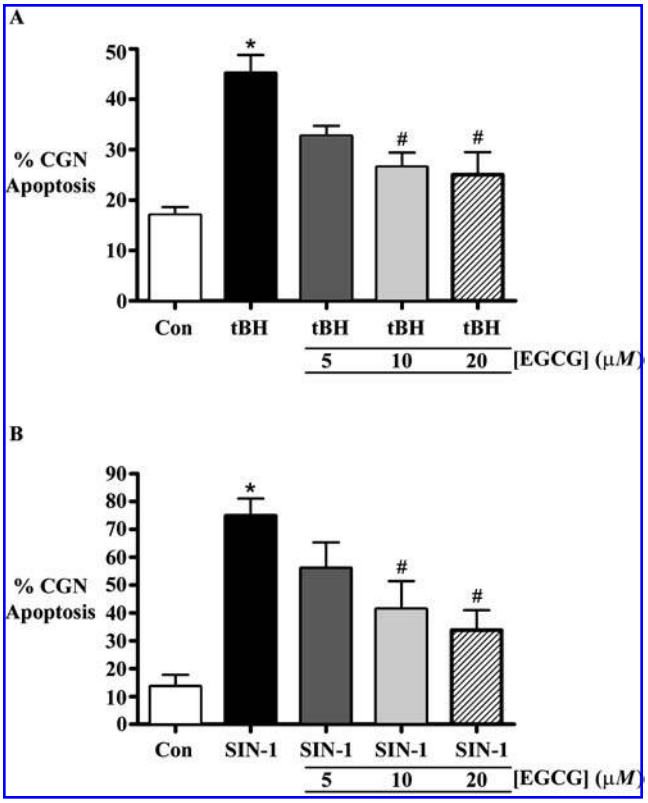


FIG. 5. CGNs treated with tBH or SIN-1 (generators of H_2O_2 and peroxynitrite, respectively) are significantly protected by co-incubation with EGCG. CGNs were incubated in either control medium containing serum with 25 m*M* potassium (Con), *tert*-butylhydroperoxide (tBH, 50 μM; **A**), or SIN-1 (1.75 m*M*; **B**). Some CGNs were co-incubated with EGCG at increasing amounts (5, 10, or 20 μM). Apoptosis was quantified after 24 h by Hoechst staining. EGCG significantly protected CGNs from these proapoptotic insults. *Significantly different from Con ($p < 0.01$); #significantly different from tBH or SIN-1 alone ($p < 0.01$).

EGCG does not significantly alter the activity or expression of proteins known to mitigate oxidative stress

SOD is a key antioxidant enzyme that converts superoxide radical into hydrogen peroxide (H_2O_2). In turn, H_2O_2 is subsequently detoxified by either catalase or GSHpx activity. Previous studies have indicated that EGCG can alter the expression or activity or both of these key antioxidant enzymes in some cell types (29, 39, 40). In contrast, CGNs incubated with EGCG (20 μM) for 24 h did not display any significant increase in the activity of either SOD or mitochondrial GSHpx (Fig. 7A–C). Given the large variability observed in the SOD-activity data (Fig. 7A), we next analyzed the expression of MnSOD, the mitochondria-specific isoform of this enzyme. EGCG treatment had no significant effect on the level of MnSOD protein detected in CGNs (Fig. 8A), further suggesting that EGCG does not markedly alter SOD activity in these cells. Finally, EGCG has been reported to increase the expression of Bcl-2 (7), as well as the Nrf2 transcription factor, which promotes the expression of various antioxidant enzymes, including MnSOD and GSHpx (39, 40). However, incubation of CGNs with EGCG for 24 h had

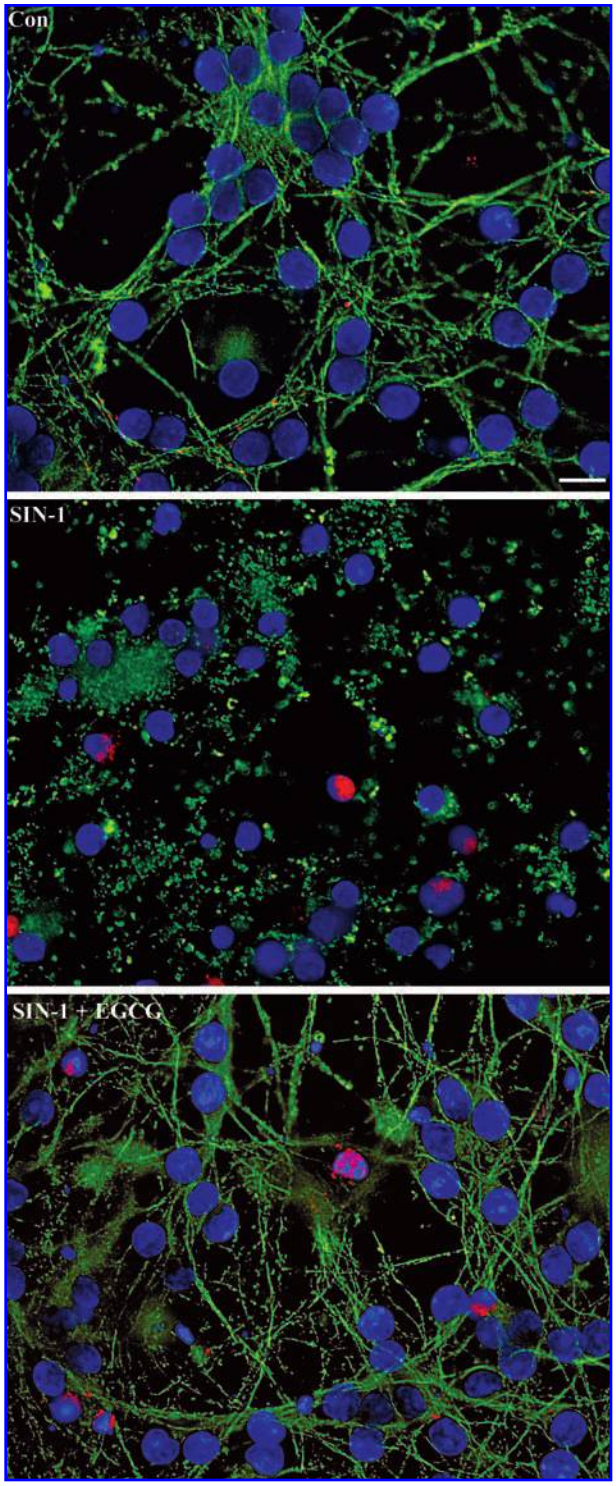


FIG. 6. EGCG protects CGNs against microtubule disruption induced by SIN-1. CGNs were incubated exactly as described in Fig. 5B. After 24-h incubation, cells were fixed and their nuclei stained with DAPI. The microtubule network was visualized by using a monoclonal antibody to β-tubulin and a FITC-conjugated secondary antibody. Active caspase-3 was detected by using a polyclonal antibody and a secondary antibody conjugated to Cy3. Significant protection and preservation of the microtubule network were observed with EGCG co-incubation (*middle panel vs. bottom panel*). Scale bar = 10 μm.

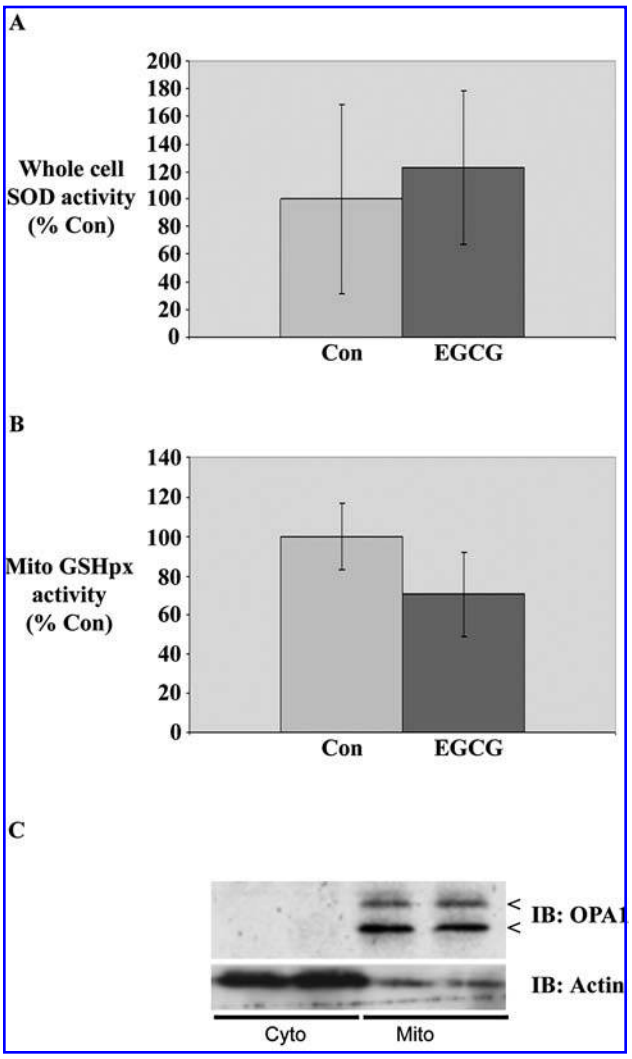


FIG. 7. EGCG treatment does not enhance SOD activity or mitochondrial GSHpx activity. CGNs were incubated for 24 h in either control medium containing 25 m*M* potassium chloride and serum (Con) alone or containing EGCG (20 μM). Whole-cell superoxide dismutase (SOD) activity was quantified as described in Materials and Methods and expressed relative to Con (**A**). Data represent the mean ± SEM of three independent experiments performed in triplicate. (**B**) CGNs were incubated exactly as described in (**A**). Mitochondrial fractions were prepared, and mitochondrial glutathione peroxidase (Mito GSHpx) activity was measured as described in Materials and Methods. Data represent the mean ± SEM of three independent experiments performed in triplicate. (**C**) CGNs were fractionated into mitochondrial and cytosolic fractions according to the protocol as described in Materials and Methods. After fractionation, proteins were resolved by SDS-PAGE, and membranes immunoblotted (IB) with antibodies to the mitochondrial marker protein, OPA-1, and β-Actin. The blot verifies the purity of the mitochondrial fractions used for the GSHpx assay in (**B**).

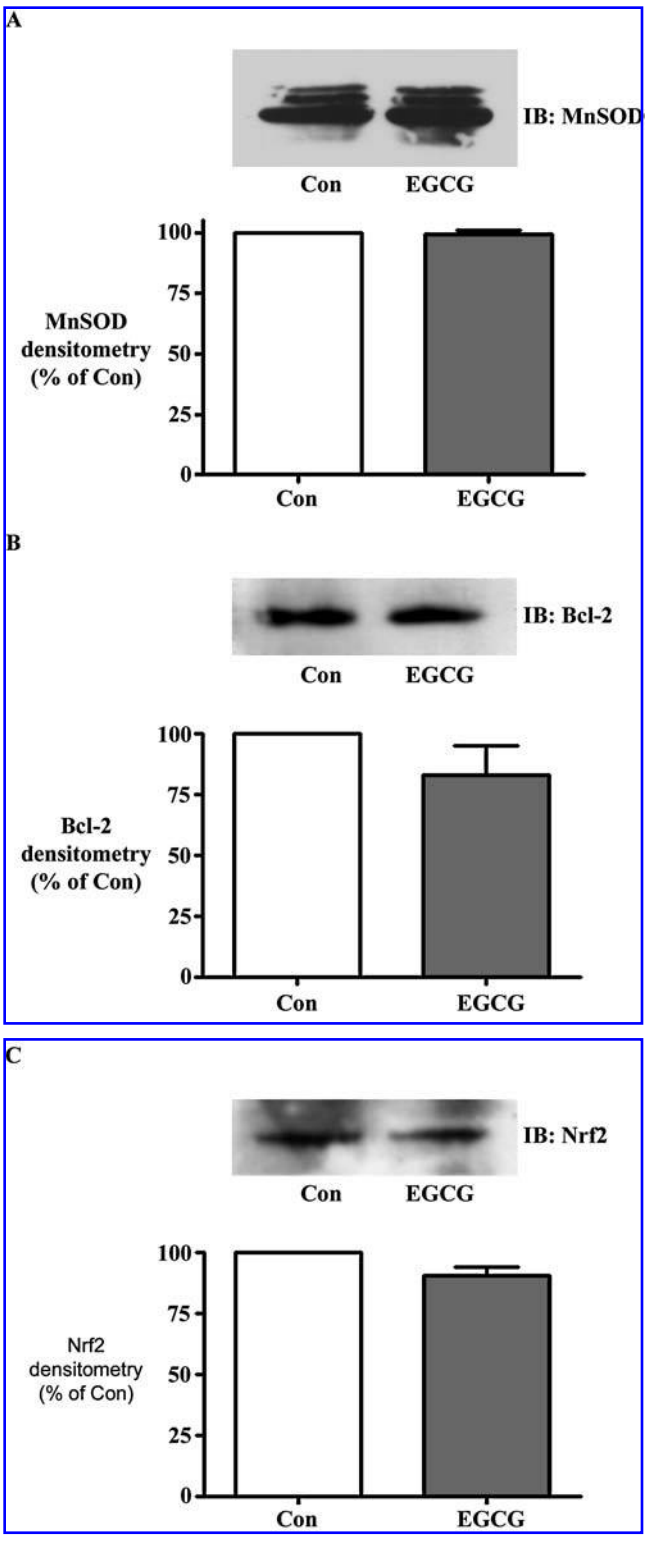


FIG. 8. ECGC does not induce antioxidant or prosurvival protein expression in CGNs. CGNs were incubated for 24 h in either control medium (Con) alone or containing EGCG (20 μM). Cells were then lysed, proteins resolved by SDS-PAGE, and membranes immunoblotted (IB) with antibodies to MnSOD (**A**), Bcl-2 (**B**), or Nrf-2 (**C**). Respective bar graphs show the mean ± SEM densitometry of three independent experiments performed in triplicate. No significant differences were observed in protein expression between the two treatments (Con or EGCG).

no detectable effect on the expression level of either Bcl-2 or Nrf2 (Fig. 8B and C). These data suggest that induction of endogenous antioxidants is not a principal mechanism by which EGCG protects CGNs from mitochondrial oxidative stress and apoptosis.

EGCG selectively accumulates in CGN mitochondria

The specificity of EGCG for protecting CGNs against mitochondrial oxidative stressors prompted us to investigate whether this catechin shows any preference for localization to mitochondria. CGNs were incubated for 24 h with a tracer amount of ^{3}H-EGCG in either the absence or presence of unlabeled "cold" EGCG (20 μM). After incubation, CGNs were subfractionated into cytosolic and mitochondrial fractions (Fig. 9C), and the distribution of labeled EGCG was determined by liquid scintillation counting. In two independent experiments, >90% of the ^{3}H-EGCG partitioned into the mitochondrial fraction (Fig. 9A). Moreover, co-incubation of CGNs with an excess of cold EGCG caused a marked 65–90% reduction in the total amount of ^{3}H-EGCG taken up by mitochondria when compared with cells incubated with ^{3}H-EGCG alone (Fig. 9B). These results demonstrate that EGCG selectively accumulates in the mitochondria of CGNs, providing a unique basis for its specific neuroprotective efficacy against inducers of mitochondrial oxidative stress.

Discussion

In the current study, we systematically analyzed the neuroprotective effects of green-tea EGCG in cultured rat CGNs. An examination of eight distinct proapoptotic insults revealed that EGCG selectively protects CGNs from apoptosis induced by mitochondrial oxidative stressors. The concentrations or incubation conditions or both for each of the eight stressors were carefully selected to yield comparable levels of CGN apoptosis (~45–70% cell death). In this manner, the relative effectiveness of EGCG to protect CGNs against any given stimulus could be compared with the other stimuli without the confounding variable of markedly different levels of cell death. Although EGCG failed to protect CGNs from several other proapoptotic stimuli, these negative findings still provide significant information about the neuroprotective mechanism of this catechin. For instance, we and others have shown that insulin-like growth factor-I rescues CGNs from 5K-induced apoptosis through the activation of a prosurvival PI3K/AKT pathway (9, 33). EGCG has been shown to activate the PI3K/AKT pathway in motoneurons and neuroblastoma × dorsal root ganglion hybrid cells (21, 22). However, the inability of EGCG to protect CGNs from 5K-induced apoptosis suggests that activation of AKT is not a common mechanism by which this catechin protects neurons. Similarly, we and others have shown that inhibition of proapoptotic JNK activity protects CGNs from apoptosis induced by 5K, MG132, and ToxB (4, 5, 24, 32). EGCG has been reported to inhibit JNK activity induced by either UV or H_2O_2 in keratinocytes (18, 20). Yet, EGCG failed to protect CGNs from either 5K, MG132, or ToxB, suggesting that it does not inhibit JNK activation induced by these proapoptotic stimuli. Finally, EGCG has been shown to inhibit glutamate-induced excitotoxicity in PC12 cells (25), but it did not protect CGNs from either glutamate or brefeldin A, an inducer of IP3 receptor–dependent Ca^{2+} release from the endoplasmic reticulum (3). Our data do not refute the results of previous studies demonstrating diverse mechanisms of EGCG-mediated cytoprotection. Instead, our findings suggest that these alternative mechanisms of action may represent cell type–specific or stimulus-specific effects of EGCG that are not universal to all cell types.

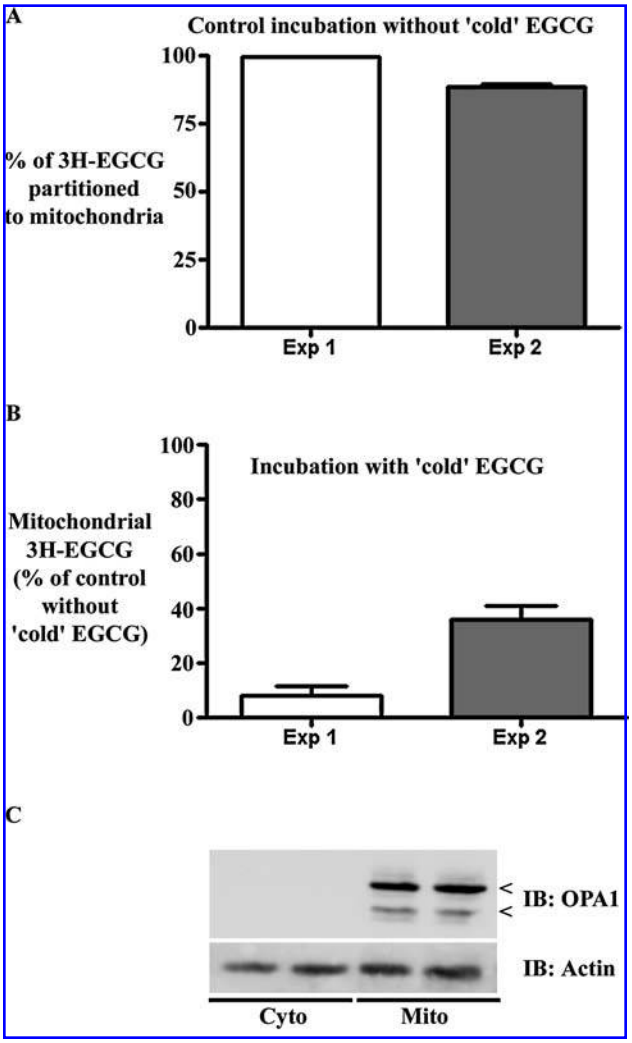


FIG. 9. EGCG accumulates in the mitochondrial fraction of CGNs. CGNs were incubated for 24 h in control medium containing 1 μCi of ^{3}H-EGCG (10 Ci/mmol) in either the absence or presence of 20 μM unlabeled ("cold") EGCG. After incubation, CGNs were lysed, and cytosolic and mitochondrial fractions were obtained by using the procedure outlined in Materials and Methods. Aliquots of cytosolic (100 μl) and mitochondrial (10 μl) fractions were counted by liquid scintillation. (**A**) Data for CGNs incubated *without* cold EGCG are presented as a percentage of the total cellular ^{3}H-EGCG that partitioned into the mitochondrial fraction. (**B**) Results for CGNs incubated *with* cold EGCG are presented as a percentage of the mitochondrial ^{3}H-EGCG observed in CGNs incubated *without* cold EGCG. (**C**) The purity of the mitochondrial fractions was verified by immunoblotting for OPA1. The data shown are the means ± SEM of triplicate wells from two independent experiments (denoted Exp 1 and Exp 2) that each produced similar results.

In contrast to these results, we found that EGCG displayed a significant protective effect against CGN apoptosis induced by a Bcl-2 inhibitor (HA14-1), tBH, or peroxynitrite (SIN-1). Each of these insults shares a common pathway to apoptosis through the induction of mitochondrial oxidative stress (38, 53, 55, 56). The selective neuroprotection observed with EGCG against these mitochondrial oxidative stressors was

unexpected, given the multifunctional properties attributed to this catechin (reviewed in 14, 35, 37). Nonetheless, our data support the hypothesis that the *intrinsic free radical scavenging activity* of EGCG is a critical element of its neuroprotective action. EGCG is known to scavenge several reactive oxygen and nitrogen species, as well as acting as a chelator of transition metals such as iron and copper (14, 35, 37). This hypothesis is further supported by the findings that EGCG did not significantly alter the expression or activity of a number of endogenous antioxidant or prosurvival proteins in CGNs. Moreover, we observed a striking and previously unreported accumulation of EGCG within the mitochondrial fraction of CGNs. The partitioning of EGCG to mitochondria was specific, as we could competitively decrease the amount of labeled EGCG recovered in the mitochondrial fraction by co-incubation of CGNs with an excess of cold EGCG. In particular, this latter finding suggests that the active accumulation of EGCG within mitochondria is probably a determining factor in its selectivity for protecting CGNs from mitochondrial oxidative stress.

Because mitochondrial oxidative stress is a key player in neurodegenerative diseases like Parkinson's and ALS (reviewed in 30), many laboratories are focused on the discovery of novel antioxidants that are selectively targeted to mitochondria. Some examples of this include mitochondria-targeted peptide antioxidants (54), mitochondria-targeted vitamin E (43), and mitochondria-targeted ubiquinone, Mito Q (19). Our findings are novel in that they are the first to show that the natural-product antioxidant, EGCG, displays an intrinsic "targeting" to neuronal mitochondria. This unique property, in combination with its innate capacity to act as a free radical scavenger, makes EGCG an interesting therapeutic candidate for the treatment of neurodegenerative disorders in which mitochondrial oxidative stress and apoptosis are major contributing factors.

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Abbreviations

ALS, Amyotrophic lateral sclerosis; APPsw, Amyloid precursor protein, Swedish mutant; Bcl-2, B-cell lymphoma 2; BH3, Bcl-2 homology-3; BrefA, brefeldin A; BSA, bovine serum albumin; CCD, charge-coupled device; CGNs, cerebellar granule neurons; Cy3, indocarbocyanine; DAPI, 4,6-diamidino-2-phenylindole; EGCG, epigallocatechin-3-gallate; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; G/G, glutamate/glycine; GSH, glutathione; GSHpx, glutathione peroxidase; H_2O_2, hydrogen peroxide; HA14-1, 2-amino-6-bromo-α-cyano-3-(ethoxycarbonyl)-4H-1-benzopyran-4-acetic acid ethyl ester; IB, immunoblotted; IP3, inositol 1,4,5-trisphosphate; JAK, Janus kinase; JNK, c-Jun-NH_2 terminal kinase; MAP, mitogen-activated protein; mito Q, mitochondria-targeted ubiquinone; MnSOD, Mn^{2+}-superoxide dismutase; MPP+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nrf2, nuclear factor E2–related factor 2; OPA1, optic atrophy type 1; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with 0.1% Tween 20; PKC, protein kinase C; Pl3K/AKT, phosphatidylinositol 3-kinase/protein kinase B; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; SIN-1, 3-morpholinosydnonimine; SOD1, Cu, Zn-superoxide dismutase; tBH, *tert*-butylhydroperoxide; ToxB, *Clostridium difficile* toxin B.

Disclosure Statement

No competing financial interests exist.

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