Abstract. It has been acknowledged that environmental stress is a risk factor for developing mental disorders. Chronic stress may contribute to the hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis and a sustained rise in the levels of glucocorticoids (GCs). A high concentration of corticosterone (CORT) damages neuronal PC12 cells. It has been reported that (-)-Epigallocatechin-3-gallate (EGCG), a major component of green tea, exhibits neuroprotective activity. However, the protective effect of EGCG on neuronal cells injured by CORT remains to be elucidated. The present study aimed to identify the effects of EGCG on CORT-injured neuronal PC12 cells and its associated mechanisms of action. CORT-injured PC12 cells were pretreated with EGCG with or without cyclopamine. Cell viability was assessed using an MTT assay, changes in cell morphology were observed using phase-contrast microscopy, cellular apoptosis was assessed by Hoechst 33342 staining, cell proliferation was measured using a cell counting kit-8 assay, mRNA levels were measured by reverse transcription-quantitative polymerase chain reaction and protein expression was assessed using western blot analysis. The current study demonstrated that exposure to high concentrations of CORT induced cytotoxicity and downregulated the Sonic hedgehog pathway (Shh) in PC12 cells. These effects were attenuated by EGCG. However, the EGCG-mediated neuroprotective effects, as well as upregulation of the Shh pathway were all attenuated by the Shh signaling inhibitor cyclopamine. These results indicate that EGCG protects PC12 cells from CORT-induced neurotoxicity via activation of the Shh signaling pathway.

Introduction

The prevalence of mental disorders such as depression has increased over the past few years and such disorders negatively affect the quality of life of many people around the world (1). It has been demonstrated that environmental stress is a risk factor for a number of mental disorders. Exposure to psychological and physiological-related chronic stress contributes to the dysfunction of hypothalamic-pituitary-adrenal (HPA) axis and causes a sustained elevation of glucocorticoid (GC) levels, which may result in the development of mental disorders (2). In vitro, a high concentration of GCs suppresses neuronal proliferation, growth and differentiation and may even inhibit neuronal cell death (3). It has been demonstrated that a number of classical antidepressants protect against the cytotoxicity induced by high concentrations of GCs in neural cells (4).

The consumption of green tea is very popular worldwide and it may exhibit beneficial pharmacological actions, including anti-oxidant, anti-carcinogenic, anti-tumor and anti-neurodegenerative effects (5-7). A number of studies have demonstrated that the green tea induces marked anti-depressive effects in animal models of depression (8,9) and that catechins, the major polyphenolic compounds found in green tea, decrease depression- and anxiety-like behaviors in a rat model induced following the injection of chronic corticosterone (CORT) (10). The catechin (-)-epigallocatechin-3-gallate (EGCG) is the primary constituent of the green tea polyphenols and exhibits the strongest biological activity, including antioxidant, anti-inflammatory and neuroprotective effects (11,12). It has been demonstrated that EGCG significantly improves chronic unpredictable mild stress-induced behavior alterations in rats (13) and exerts neuroprotective effects against...
different types of stress-related damage, including trauma and neuronal damage induced by L-3,4-dihydroxyphenylalanine (L-DOPA) (14,15). However, few studies have been completed to assess the effect of EGCG on neural cells damaged following exposure to high concentrations of CORT.

The adrenal phaeochromocytoma PC12 cell line is one of the most widely used neuronal cell lines and is commonly used to study the neuronal damage induced by GCs in vitro (16) and also used as an in vitro experimental model of depression (17,18). Although previous studies have suggested that EGCG has neuroprotective effects, the effect of EGCG on neuronal cells exposed to high concentrations of CORT remains to be elucidated. The present study examined the neuroprotective activity and associated potential mechanisms of EGCG in CORT-injured PC12 cells.

Materials and methods

Materials. The PC12 cell line was supplied by the Central Laboratory of the Central Hospital of Wuhan (Wuhan, China). The RPMI 1640 medium was purchased from Hyclone; GE Healthcare Life Sciences (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biotechnology Co., Ltd. (Hangzhou, China). EGCG (>97.0%) was purchased from Selleck Chemicals (Houston, TX, USA). CORT (>97.0%) and the hedgehog-smoothened inhibitor cyclopamine (>99.0%) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Desipramine (DIM; >98.0%), a well-known antidepressant (19) which was used as the positive control (18) in the present study, was purchased from Sigma Aldrich; Merck KGaA (Darmstadt, Germany). All pharmacological agents were prepared as a stock solution and stored at -20°C.

Cell culture and treatment. PC12 cells were maintained in 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO₂ atmosphere at 37°C. Cells were seeded at a density of 8x10⁵ cells/well in 96-well plates and incubated for 2-3 days for MTT assay; cells were seeded at a density of 4x10⁵ cells/well in 96-well plates and incubated for 1 days for the CCK8 assay. The cells were divided into five groups: Control group, where PC12 cells were not treated; CORT group, where PC12 cells were treated with CORT; CORT+EGCG group, where PC12 cells were treated with CORT and EGCG, and EGCG was added 1 h before CORT; CORT+DIM group, where PC12 cells were treated with CORT and DIM, and DIM was added 1 h before CORT; and Cyclopamine+EGCG+CORT group, where PC12 cells were treated with CORT, EGCG and cyclopamine, and cyclopamine was added 30 min before EGCG, and then EGCG was added 1 h before CORT.

MTT assay. A 3-(4,5-Desethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Biosharp, Inc., Pivotal Scientific, Wuhan, China) assay was used to measure cell viability. Cells were seeded at a density of 8x10⁵ cells/well in 96-well plates and cultured, and following incubation of the cells with the different drugs for 24 h, they were treated with the MTT solution and incubated at 37°C for 4 h. The dark blue formazan crystals that formed in the wells were solubilized with dimethyl sulfoxide for 10 min at 37°C. Absorbance at 570 nm was measured using a microplate reader (PerkinElmer, Inc, Waltham, MA, USA).

Morphological changes. Following treatment of PC12 cells with different drugs, grown medium was removed by PBS. Cellular morphology was observed using a fluorescence microscope (BX-50-FLA, Olympus Corporation, Tokyo, Japan).

Hoechst 33342 staining. Cells were treated with different drugs and incubated for 24 h. The medium was subsequently replaced by Hoechst 33342 (Beyotime Biotechnology Institute of Biotechnology, Nanjing, China) solution and incubated at 37°C for 15 min. Cells were then observed under an inverted fluorescence microscope (BX-50-FLA, Olympus Corporation). Apoptotic cells exhibited strong blue fluorescence and shrunken nuclei, whereas non-apoptotic cells exhibited weak blue fluorescence and normal nuclei.

Cell counting kit-8 (CCK-8) assay. A CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to measure cell proliferation. Cells were seeded and cultured in 96-well plates and after the cells were treated with the different drugs and incubated for 0, 12, 24, 36, 48 and 60 h, the medium was replaced with CCK-8 reagent (Dojindo Molecular Technologies, Inc.) and cells were incubated for a further 2 h at 37°C. Finally, the optical density of each well was measured using a microplate reader at a wavelength of 450 nm. The percentage of the control samples of each cell line was calculated thereafter.

Preparation of total RNA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were treated with the aforementioned drugs and cultured for 24 h. Subsequently, cells were harvested and total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The purity of total RNA was evaluated using the optical density 260/280 ratio and a UV spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). RNA purity was determined to be between 1.7 and 2.0.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to determine mRNA expression of proteins in the hedgehog signaling pathway. Reverse transcription was performed using cDNA Synthesis kit (Toyobo, Co., Ltd., Osaka, Japan) following the manufacturer's protocol. cDNA was subjected to qPCR using the Power SYBR® Green PCR Master mix (CWBIOTECH, Beijing, China). The specific sequences of the primers used were as follows: GAPDH, forward 5'-GATGGTGAAAGTTGGTGTA-3' and reverse 5'-GTCATGAAAGGGTGTTGTA-3'; Shh, forward 5'-TATGGGTCGACGGATGA-3' and reverse 5'-AGTGGAATGCGAGCTTTGAT-3'; Gli1, forward 5'-GTCACTACCTGGCCATCACA-3' and reverse 5'-CCCTGATTGTTTGTAAT-3'; and N-myc, forward 5'-GATGACTTTCTACTTGCGTGTA-3' and reverse 5'-TTTGCTACACCCTTCGGTG-3'. qPCR was performed under the following conditions: 95°C for 30 sec, followed by 45 cycles at 95°C for 5 sec, 60°C for 30 sec in the ABI 7900 Real-Time PCR System (Applied Biosystems;
Thermo Fisher Scientific, Inc.). The relative quantification of gene expression was calculated using the 2^{-\Delta\Delta Cq} method (20). Each gene analysis was performed in triplicate.

Western blot analysis. Cells were lysed with ice-cold radioimmunoprecipitation assay lysis buffer with 10% phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology, Haimen, China) and the protein was collected after being centrifuged at 12,000 x g for 30 min at 4°C. Protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology) following the manufacturer’s protocol. Equal amounts of protein (20 µg per lane) were electrophoresed on 10% density SDS acrylamide gels and then proteins were transferred to nitrocellulose membranes. Non-specific binding was blocked with 5% skim milk in TBST buffer at 4°C for 2-3 h, prior to incubation with rabbit anti-N-myc antibody (1:200, sc-791; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and rabbit anti-GAPDH antibody (1:1,000, sc-25778; Santa Cruz Biotechnology, Inc.,) overnight at 4°C. Membranes were then incubated with FITC-conjugated goat anti-rabbit IgG secondary antibody (1:10,000, BL033A; Biosharp Inc., Pivotal Scientific) for 1 h at room temperature. Finally, detection was performed using enhanced chemiluminescence (GE Healthcare Life Sciences) and quantified using Quantity One 4.6.2 imaging software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data were expressed as the mean ± standard error of the mean. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Prism 5.0 software (GraphPad software, Inc., La Jolla, CA, USA) to detect any inter-group differences. P<0.05 was considered to indicate a statistically significant difference.

Results

EGCG protects against the cytotoxicity induced by CORT. Differentiated PC12 cells were treated with 50, 100, 150, 250, 400 µM CORT for 24 h. Subsequently, the effect of CORT on PC12 cell viability was determined using a MTT assay. Different concentrations of CORT decreased cell viability in a dose-dependent manner (Fig. 1A). The concentration of 400 µM CORT, which significantly decreased cell viability by 35.6% (P<0.01; Fig. 1A) was selected for subsequent experiments. The protective effects of different concentrations of EGCG was subsequently investigated. EGCG induced no cytotoxic effects at concentrations between 1 and 20 µM (Fig. 1B), although concentrations of EGCG >30 µM significantly decreased cell viability compared with the control (P<0.05). It was also demonstrated that EGCG attenuated the decrease in cell viability induced by CORT in a dose-dependent manner and that the activity of EGCG was optimal at a concentration of 20 µM (Fig. 1C). The viability of PC12 cells treated with 20 µM EGCG and 400 µM CORT increased significantly by 20.1% compared with the group treated with 400 µM CORT alone (P<0.01; Fig. 1C). DIM significantly increased the viability of PC12 cells (P<0.05) at a concentration of 1 µM compared with the group treated with 400 µM CORT alone (Fig. 1D). To determine the protective effect of EGCG on CORT-injured PC12 cells, the morphological changes of PC12 cells treated with different drugs were observed using an inverted microscope (Fig. 1E). The morphology of cells treated with 400 µM CORT changed and the axons shrunken markedly. However, these alterations were attenuated when cells in the 400 µM CORT group were pretreated with 20 µM EGCG or 1 µM DIM. Thus, EGCG may protect PC12 cells against the cytotoxicity induced by CORT.

EGCG attenuates CORT-induced cell apoptosis and inhibition of cell proliferation. Cellular apoptosis induced by different drugs was detected by staining with Hoechst 33342 (Fig. 2A). The results demonstrated that treatment with 400 µM CORT markedly increased the amount of condensed chromatin in PC12 cells. The addition of 20 µM EGCG decreased the amount of condensed chromatin compared with the CORT

Figure 1. Protective effect of EGCG against the cytotoxicity induced by CORT in PC12 cells. (A) The viability of PC12 cells treated with 50, 100, 150, 250, 400 µM CORT was measured using an MTT assay 24 h after treatment of cells with CORT. (B) The viability of PC12 cells treated with 1, 10, 20, 30, 50 µM EGCG was measured using an MTT assay 24 h after incubation with 400 µM CORT and incubated for 24 h prior to an MTT assay. (C) The viability of PC12 cells treated with 1, 5, 10, 15, 20 µM EGCG and 400 µM CORT was measured using an MTT assay. PC12 cells were pretreated with different concentrations of EGCG 1 h before treatment with 400 µM CORT and incubated for 24 h prior to the MTT assay. (D) The viability of PC12 cells treated with 0.1, 0.5, 1, 5, 10 µM DIM (positive control) and 400 µM CORT was measured using an MTT assay. PC12 cells were pretreated with different concentrations of DIM 1 h before incubation with 400 µM CORT for 24 h prior to an MTT assay. (E) Images of cell morphology were taken 24 h after the cells were treated with 400 µM CORT alone, 400 µM CORT and 20 µM EGCG or 400 µM CORT and 1 µM DIM (magnification, x200). All results are presented as the mean ± standard error of the mean of three independent experiments performed in triplicate. *P<0.05 and **P<0.01 vs. control; #P<0.05 and ##P<0.01 vs. CORT group. EGCG, (-)-Epigallocatechin-3-gallate; CORT, corticosterone; DIM, desipramine.
The optical density (OD) of PC12 cells treated with 20 µM EGCG and 400 µM CORT was determined using a CCK8 assay. Compared with the control group, the OD of the cells were significantly decreased when treated with 400 µM CORT for 12 (P<0.05), 24 (P<0.001), 36 (P<0.001), 48 (P<0.001), 60 h (P<0.001) vs. control; *P<0.05, **P<0.01 and ***P<0.001 vs. CORT group. EGCG, (-)-Epigallocatechin-3-gallate; CORT, corticosterone; CCK-8, cell counting kit-8.

Figure 3. The effect of EGCG on the CORT-induced downregulation of Shh signaling. PC12 cells were treated with 20 µM EGCG 1 h prior to treatment with 400 µM CORT. mRNA and protein levels were measured 24 h after treatment with 400 µM CORT. (A and B) Relative mRNA expressions of Shh, Gli1, N-myc were measured by reverse transcription-quantitative polymerase chain reaction following treatment with (A) 400 µM CORT or 20 µM EGCG or (B) 400 µM CORT and 20 µM EGCG. (C) The expression of N-myc protein was measured using western blot analysis. The results are presented as the mean ± standard error of the mean of three independent experiments performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs. control; *P<0.05 vs. CORT group. Shh, Sonic hedgehog pathway; EGCG, (-)-Epigallocatechin-3-gallate; CORT, corticosterone.
EGCG contributes to the activation of Shh pathway in CORT-treated PC12 cells and this signaling may be involved in the protection of EGCG in CORT-injured PC12 cells. The Shh signaling pathway is involved in the neuroprotective effect of EGCG. To identify the role of Shh signaling pathway in the protective effect of EGCG, prior to treatment with 20 µM EGCG and 400 µM CORT, cells were pretreated with 20 µM cyclopamine, which targets the Sonic hedgehog signaling pathway by blocking the activity of the smoothened frizzled class transmembrane receptor (SMO). The results demonstrated that, compared with the EGCG + CORT group, pretreatment with 20 µM cyclopamine significantly decreased the cell viability (P<0.01; Fig. 4A), although it could not decrease the cell viability to a similar level in the CORT group (Fig. 4B). This suggests that EGCG contributes to the activation of Shh pathway in CORT-treated PC12 cells and this signaling may be involved in the protection of EGCG in CORT-injured PC12 cells.

The Shh signaling pathway is involved in the protective effect of EGCG. To identify the role of Shh signaling pathway in the protective effect of EGCG, prior to treatment with 20 µM EGCG and 400 µM CORT, cells were pretreated with 20 µM cyclopamine, which targets the Sonic hedgehog signaling pathway by blocking the activity of the smoothened frizzled class transmembrane receptor (SMO). The results demonstrated that, compared with the EGCG + CORT group, pretreatment with 20 µM cyclopamine significantly decreased the cell viability (P<0.01; Fig. 4A), although it could not decrease the cell viability to a similar level in the CORT group (Fig. 4B). This suggests that EGCG contributes to the activation of Shh pathway in CORT-treated PC12 cells and this signaling may be involved in the protection of EGCG in CORT-injured PC12 cells.

Discussion

Supraphysiological GC levels may have detrimental effects on neurodevelopment (21) and a significant chronic rise in GC levels induced by stress is associated with pathological changes in the hippocampus, which contributes to the pathogenesis of...
depression. Previous studies have demonstrated that exposure to high levels of GCs may suppress neuronal proliferation, growth and differentiation and lead to the death of neuronal cells, including PC12 cells, in vitro (3,22). Consistent with these observations, the present study demonstrated that treatment with 400 µM CORT induced a significant decrease in cell survival and growth as well as the shrinking of cells, confirming that CORT induces neurotoxicity. EGCG alleviated cytotoxicity induced by CORT but had no effect on normal cells, indicating that EGCG reverses CORT-induced neuronal damage.

The present study demonstrated that EGCG increases the viability and proliferation of PC12 cells in vitro following CORT injury and that treatment with 20 µM induces the greatest effects. Higher doses of EGCG, such as 100 µM EGCG, are harmful to normal PC12 cells, thus confirming the hypothesis that the biological activity of EGCG follows a characteristic biphasic pattern: Higher doses of EGCG exhibit pro-oxidant and pro-apoptotic effects, whereas lower doses of EGCG have neuroprotective effects (23,24). Previous studies have suggested that EGCG has a neuroprotective function. It has been demonstrated that EGCG protects the brain against traumatic injury (14), inhibits the oxidative stress induced by 1-methyl-4-phenylpyridinium in neuronal cells (25), attenuates the neurotoxicity induced by L-DOPA or oxygen-glucose deprivation (15,26) and promotes the neurogenesis of adult hippocampus in vivo and in vitro (27). The current study confirmed that EGCG protects neuronal cells against CORT-induced damage, indicating that EGCG is neuroprotective.

However, the precise cellular and molecular mechanisms underlying CORT-induced neurotoxicity are not fully understood. It has been demonstrated that in prenatally stressed rats and hippocampus neural cells treated with high concentrations of cortisol, the inhibition of Hedgehog (Hh) signaling by high concentrations of GCs contributes to neurotoxicity (28) and that activation of Shh signaling is protective against GC-induced neurotoxicity (29,30). Hh signaling serves an important role in neuronal development and growth, as well as the repair of damage (31) and is activated by the binding of secreted Hh proteins, including Shh, Desert hedgehog and Indian hedgehog, to the transmembrane receptor Patched (Ptc). Upon binding to Ptc, the Hh protein reverses the repression of the transmembrane receptor SMO, thus activating the major transcription factor Gli that regulates the expression of genes, such as those encoding N-myc, to influence the survival, growth and differentiation of cells (32). The present study demonstrated that Gli1 and N-myc were inhibited in PC12 cells by high concentrations of CORT, which is similar to the results of studies that indicated chronic GC treatment reduced the expression of Gli1 and N-myc in neonatal brain or medulloblastoma (30,33). Furthermore, consistent with the results of a study by Heine et al (30), the current study did not detect any changes in Shh expression, demonstrating that CORT acted downstream of Shh in this pathway and that Gli1 may be a target of CORT.

Activation of Shh signaling is involved in protection against neuronal damage (34), however little is known regarding its role in EGCG-enhanced protection following CORT-induced neurotoxicity. EGCG stimulates intracellular signaling pathways associated with cell survival and growth and Shh signaling is involved in EGCG-mediated neuroprotection (35). In the current study, it was determined that pretreatment with EGCG attenuated the CORT-induced downregulation of Gli1 and N-myc, implying that EGCG may contribute to the activation of the Shh signaling pathway. By contrast, when cells were treated with the Shh inhibitor cyclopamine prior to EGCG, the increase in cell viability and proliferation was reversed. Furthermore, the expression of Gli1 and N-myc were suppressed by cyclopamine, indicating that Shh signaling contributes to the protective effect of EGCG. Although cyclopamine reversed the effect of EGCG on promoting cell survival, it could not decrease the cell viability to a similar level with the CORT group. This suggested that the increase in cell viability was not completely blocked by cyclopamine, indicating that Shh signaling may not be the only mechanism by which EGCG acts (13). Therefore, further studies are required to elucidate these other potential mechanisms of action.

In conclusion, the current study demonstrated that EGCG exhibits protective activities and contributes to the activation of the Shh signaling pathway in CORT-injured PC12 cells. Furthermore, Shh signaling may be involved in the neuroprotective effects of EGCG against CORT-induced neurotoxicity. The results of the current study may be useful in improving understanding regarding the neuroprotective effects of the various compounds contained in green tea. However, further experimental studies in vivo are required to examine the effects of EGCG and other compounds in green tea on stress-related neurotoxicity and neurological disorders.

Acknowledgements

The current study was supported by the National Natural Science Foundation of China (grant no. 81603013), the Basic Research of Wuhan Science and Technology Bureau (grant no. 2017060201010204) and the Chenguang Plan of Wuhan Municipal Science and Technology Bureau (grant no. 201407040401010226).

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