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ORIGINAL ARTICLE

Protective effects of ethyl gallate on H_2O_2-induced mitochondrial dysfunction in PC12 cells

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Abstract

Oxidative stress has been suggested to play an important role in neuronal injury. Ethyl gallate (EG) is the ethyl ester of gallic acid which has been acknowledged as an antioxidant. We previously demonstrated that EG effectively inhibited H_2O_2-induced cytotoxicity and decreased the ROS levels in PC12 cells, while the relevant mechanisms of action of this compound remain largely uncharacterized. The present study was carried out in an attempt to clarify the underlying mechanisms of EG against H_2O_2-induced neurotoxicity in PC12 cells. EG pretreatment attenuated H_2O_2-induced mitochondrial dysfunction as indicated by the decreased caspase-9/−3 activation, PARP cleavage, mitochondrial membrane potential (MMP) depletion, Bax/Bcl-2 ratio, cytochrome *c* release and ROS overproduction. Furthermore, EG treatment resulted in nuclear translocation of Nrf2 along with increased expression of ARE-dependent cytoprotective genes, such as γ-GCS and NQO1, which indicated EG as an Nrf2 pathway activator. Silencing of Nrf2 signaling by siRNA abrogated the protective effects offered by EG on H_2O_2-induced PC12 cells injury, which suggested the important role of Nrf2 pathway in the protection of EG against oxidative stress induced PC12 cell apoptosis. These results taken together indicated that EG protects PC12 cells against H_2O_2-induced cell mitochondrial dysfunction possibly through activation of Nrf2 pathway. EG might be a potential candidate for further preclinical study aimed at the prevention and treatment of neurodegenerative diseases.

Keywords Ethyl gallate · Mitochondrial dysfunction · Nrf2 pathway · PC12 cells

Introduction

Neurodegenerative diseases are a group of disorders that are characterized by the progressive loss of structure or function of neurons (Zhang et al. 2017b). With the growing of aging population worldwide, more people are getting suffered from these age-related neurodegenerative diseases. Although pharmacological treatments available today may help relieve some of the physical or mental symptoms, neurodegenerative diseases are still incurable (Clark 2008). Increased oxidative stress has been thought as one of the main causes in all neurodegenerative diseases (Emerit et al. 2004; Niedzielska et al. 2016). Thus, using antioxidants to act against oxidative stress and its associated effects on neurons may be potential therapeutic strategies (Gilgun-Sherki et al. 2001).

Natural products are rich sources of antioxidants. *Phyllanthus emblica* is a medicinal plant with distribution in tropical and subtropical areas of China, Thailand, India and the Malaysia. All parts of the plant, particularly the fruit, have been used in the herbal preparations to treat a variety of ailments (Yadav et al. 2017). In our continuous research on antioxidants, the constituent of the fruit of *P. emblica* has been studied. The study on the dried fruit of *P. emblica* yielded 25 compounds, among which, ethyl gallate (EG) is the most promising compound that improved the survival of PC12 cells after H_2O_2 exposure (Zhang et al. 2016). Rat pheochromocytoma PC12 cell line is suitable model for studying oxidative stress induced neuronal injury. Although EG has been reported to possess a range of biological activities including inhibition of nitric oxide production (Park et al. 2011), attenuation of cardiovascular dysfunction (Mink et al. 2011), attenuation of acute lung injury (Mehla et al. 2013), inhibition of cell adhesion molecules production (Mehla et al. 2011) and inducing cancer cells apoptosis (Kim et al. 2012), while the relevant mechanisms of action of the compound has not been characterized. In this present study, we focused on clarifying the underlying mechanisms of EG against H_2O_2 induced neurotoxicity in a PC12 cell neuronal model.

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Mitochondria are known as essential organelles involved in various cellular processes. Besides the production of energy in the form of ATP, mitochondria are also participating in the regulation of cell survival and death (Green and Reed 1998). Increasing evidences have supported the involvement of mitochondrial dysfunction and oxidative damage in the pathogenesis of neurodegenerative diseases (Lin and Beal 2006). The overproduction of reactive oxygen species (ROS) due to the imbalance between the generation and detoxification may lead to depolarization of mitochondrial membrane potential (MMP), subsequently trigger cytochrome *c* release from mitochondria to the cytoplasm, eventually promote neuronal cells apoptosis (Wang 2001).

In response to oxidative stress, cells have evolved antioxidant defense system to allow them survive. Nuclear factor erythroid-derived 2-related factor 2 (Nrf2) is known as a master regulator of a major cellular defense mechanism due to its ability to eliminate harmful reactive species and maintain intracellular redox balance (Lee and Johnson 2004). To counteract oxidative stress, Nrf2 up-regulates the expression of a battery of detoxification enzymes and antioxidant proteins. Nrf2 also supports the structural and functional integrity of mitochondria, particularly under conditions of stress (Dinkova-Kostova and Abramov 2015). Activation of Nrf2 pathway has been demonstrated to provide beneficial effects for neurodegeneration (de Vries et al. 2008), thus, Nrf2 pathway is being increasingly considered as a possible therapeutic target for neurodegenerative disease.

In the current study, we focused on investigating the protective effects of EG against H_2O_2 induced mitochondrial apoptosis in PC12 cells. We also identified the important role of Nrf2 signaling in the protection of PC12 cells under oxidative stress. The results of the study provide convincing experimental evidence that EG might act as a protector for oxidative stress-induced neuronal death.

Materials and methods

Materials

Ethyl gallate (EG) was purified from the fruit of *Phyllanthus emblica* by our research group, the purity was confirmed to be >98% by HPLC analysis. Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). The antibodies for Nrf2, NQO1, γ-GCS, caspase-3 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). β-actin, Bax, Bcl-2, Bcl-xL, cytochrome *c*, caspase-9 and cleaved caspase-3 were purchased from Proteintech Group (Wuhan, China). PARP was purchased from Cell Signaling Technology (Beverly, MA, USA). Alexa Fluor 594 anti-rabbit IgG antibodies were from Proteintech Group. tBHQ (Tert-butylhydroquinone) was purchased from Alfa Aeasar (Shanghai, China).

Cell culture and treatments

Rat adrenal pheochromocytoma PC12 cells were obtained from the Department of Neurobiology, Shandong University. The cells were maintained in DMEM medium containing 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in a humidified incubator containing 5% CO_2. EG was dissolved in dimethyl sulfoxide (DMSO) at 40 mM as stock solution.

MTT assay

The cell viability of PC12 cells were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay (Li et al. 2014). In brief, cells were seeded in 96-well plate at 8×10^3 cells per well. After pretreating with different concentration of EG (5, 10, 20, 40 μM) for 4 h, cells were exposed to 400 μM H_2O_2. After 24 h treatment, 10 μL 5 mg/ml MTT solution was added and incubated for an additional 4 h at 37 °C. Then the cells were lysed in dimethyl sulfoxide (DMSO), the absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Model 680, USA). In order to get means and standard deviations, each sample used triplicate wells, and at least three times were repeated for the experiments.

DAPI (4, 6-diamidino-2-phenylindole) staining

DNA condensation was detected with DAPI staining. Briefly, cells were seeded in 24-wells plate and incubated for 16–24 h, then treat cells with the relevant substances, after fixation, cells were incubated with 2.5 μg/ml DAPI (Genview Scientific Inc., USA) solution for 10 min in the dark. After washing with PBS, images of cell nuclei were taken by a flurescence microscope system (Olympus, IX71, Japan) (Chou et al. 2010).

Apoptosis assays

Apoptosis was analyzed by flow cytometry using an Annexin V-FITC/PI double staining kit (BD Biosciences, San Jose, CA, USA). PC12 cells were seeded in 6-well plates (1×10^5 cells per well) and did the same treatment as MTT assay, at the end of incubation, cells were collected, washed with PBS and centrifuged. After adding Annexin V-FITC (5 μL) and PI (5 μL), cells were incubated in the dark at room temperature for 15 min. The apoptotic rates were measured with a FACS Calibur flow cytometer (Becton Dickinson, USA) (Zou et al. 2016).

Measurement of intracellular ROS

Reactive oxygen species (ROS) levels were measured using a fluorescent probe, 2,7-dichlorodihydro fluoresceint diacetate (DCFH-DA, Sigma). PC12 cells were pretreated with EG for 4 h and then exposed to 400 μM H_2O_2 for additional 12 h. After treatment, cells were washed with PBS, and then incubated with 10 μM of DCFH-DA in medium at 37 °C for 30 min. Subsequently, cells were harvested and diluted to a density 1×10^6 cells/mL approximately. Analysis was conducted using flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm (Mehri et al. 2012).

Analysis of mitochondrial membrane potential (MMP)

The effects of EG on the MMP were analyzed by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl-carbocyanine iodide (JC-1) staining. PC12 cells were seeded in 6-well plates, after treatment with different EG, H_2O_2 or in combination, cells were washed and incubated with 2 μM of JC-1 in medium for 30 min at 37 °C (Pan et al. 2017). At the end of treatment, cells were washed with PBS and were directly monitored under a fluorescence microscope.

Measurement of intracellular glutathione (GSH) levels

A commercial reduced glutathione assay kit (Nanjing Jiancheng Bioengineering Institute, China) was used for the intracellular reduced GSH levels measurement (Lou et al. 2012). All the procedures were conducted under the manufacturer's protocol. T-BHQ was used as positive control.

Immunofluorescence

After PC12 cells were attached on glass cover slips, EG (20, 40 μM) was added into the medium and treated cells for 4 h. At the end of incubation, cells were fixed in methanol, then introduced Nrf2 primary antibody (1:100) and incubated at 4 °C overnight. Afterwards, incubated cells with secondary antibody Alexa Fluor 594 conjugated at room temperature for 1 h. DAPI (2.5 μg/ml) was used to counterstain the cell nuclei (Huang et al. 2014). Fluorescent images were taken with a fluorescence microscope.

Mitochondrial fractionation

A mitochondria isolation kit (Beyotime, China) was used for the isolation of mitochondria. In brief, PC12 cells were grown in D-100 dishes, at the end of treatment, cells were harvested and transferred cell suspension into an ice-cold Dounce homogenizer. Afterwards, the mitochondrial fraction of cells was obtained from the homogenate by the use of centrifuge (Huang et al. 2017).

Transfection of siRNA

Nrf2 siRNA and negative control siRNA were purchased from Qiagen (Valencia, CA). Transient transfection of siRNA was performed according to manufacturer's instruction using Lipofectamine 2000 reagent (Invitrogen). After PC12 cells were grown into 80% confluence, Nrf2 siRNA or scrambled random sequence negative control siRNA were transfected (Sun et al. 2018). After transfection, cells were treated with EG for 4 h and then exposed to 400 μM H_2O_2 for 24 h. Cells samples were collected for western blotting analysis or MTT assay.

Immunoblot analysis

After treatments, cell lysate was prepared by using a sample buffer (50 mM Tris-HCl [PH 6.8], 2% SDS, 10% glycerol, 100 mM DTT, 0.1% bromophenol blue). Equal amounts of proteins were loaded and electrophoresed on SDS PAGE gel followed by a transfer onto nitrocellulose membrane (Millipore). After blocking, the membrane was incubated with a diluted primary antibody solution at 4 °C overnight, and then the membrane was exposed to a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Signals were visualized using the enhanced chemiluminescence (ECL) detection reagents (Millipore, Billerica, MA, USA). β-actin was used as an internal reference (Zhao et al. 2016).

mRNA extraction and real-time quantitative reverse transcription-polymerase chain reaction

A Trizol reagent (Invitrogen) was used to extract the total mRNA of cells. Equal amount of RNA was reverse transcribed to cDNA using the PrimeScript RT reagent Kit (Takara) according to the manufacturer's instruction. Quantitative real-time PCR (qRT-PCR) was performed using a SYBR Premix Ex Taq Kit (Takara) and the condition was an initial cycling for 2 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 15 s at 55 °C and 20 s at 72 °C. The data presented are relative mRNA levels normalized to GAPDH, the value from the untreated group was set as 1. The primer sequences are list in the Table 1 (Wang et al. 2008).

Statistical analysis

Results are presented as the mean ± standard deviation (SD). To determine the significant difference between two groups,

Table 1 Primer sequences used in the RT-PCR

	primer sequences
Nrf2	forward (ACACGGTCCACAGCTCATC)
	reverse (TGTCAATCAAATCCATGTCCTG)
GCLM	forward (GACAAAACACAGTTGGAACAGC)
	reverse (CAGTCAAATCTGGTGGCATC)
NQO1	forward (ATGTATGACAAAGGACCCTTCC)
	reverse (TCCCTTGCAGAGAGTACATGG)
GAPDH	forward (CTGACTTCAACAGCGACACC)
	reverse (TGCTGTAGCCAAATTCGTTGT)

one way analysis of variance (ANOVA) test and post hoc multiple comparison Bonferroni test were used. $p < 0.05$ was considered to be significant.

Results

EG protected PC12 cells against H_2O_2-induced apoptosis and alleviated H_2O_2-induced oxidative stress

The results of cell morphology, MTT assay, Annexin V/PI staining and ROS detection reported here were consistent with our previous results (Zhang et al. 2016). In brief, pretreatment with different doses of EG (10, 20, 40 μM) inhibited the decrease of cell viability induced by H_2O_2, attenuated H_2O_2-induced cell morphological changes, decreased H_2O_2-induced cell apoptosis and inhibited the increase of intracellular ROS induced by H_2O_2 in PC12 cells (Fig. 1 b, c, d, f). With the use of DAPI staining, the anti-apoptotic effects of EG were also characterized. As shown in Fig. 1h, after treatment with 400 μM of H_2O_2 for 24 h, nuclear fragmentation and DNA condensation occurred, while pretreatment with EG inhibited these apoptotic features.

EG inhibited the decrease of MMP and the release of cytochrome *c* induced by H_2O_2 in PC12 cells

MMP reflects the function of mitochondria of cells. In order to understand the effects of EG on mitochondria, the MMP was assessed by JC-1 assay. In healthy cells with high MMP, JC-1 aggregates show intense red fluorescence, while in unhealthy cells, JC-1 remains in monomers, which show green fluorescence. The ratio of red and green fluorescence reflects the change of MMP (Brooks et al. 2013). As shown in Fig. 2a, the positive control, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) treated group showed strong green fluorescence, which indicated the collapse of MMP. Exposure of PC12 cells to H_2O_2 resulted in an increase of green fluorescence, indicating the loss of MMP. While in EG pretreated cells, a higher red/green fluorescence intensity ratio was observed, which indicated the protective effects of EG on H_2O_2 induced MMP reduction.

The release of cytochrome *c* has been linked to the decline of MMP. By using western blot analysis, the protein levels of cytochrome *c* in both cytoplasm and mitochondria were detected. As shown in Fig. 2b, 400 μM of H_2O_2 treatment induced the translocation of cytochrome *c* from mitochondria into cytoplasm, evidenced by the increased cytochrome *c* level in cytoplasm and a corresponding decreased level in mitochondria. Pretreatment with EG inhibited H_2O_2 induced cytochrome *c* translocation, while EG treatment alone did not affect the cytochrome *c* release.

EG pretreatment inhibited the activation of caspase-9 and caspase-3 induced by H_2O_2 in PC12 cells

The translocation of cytochrome *c* from mitochondria into cytoplasm is an onset event in the apoptotic process. Once in cytoplasm, cytochrome *c* binds with its cytosolic partner Apaf-1 to form complex which could recruit caspase-9 and induce procaspase-9 activation (Li et al. 1997). The activated caspase-9 then activates other downstream caspase such as caspase-3, which play a central role in the execution of the apoptotic program (Porter and Jänicke 1999). In this study, the levels of caspase-9 and -3 were determined by western blot analysis. As shown in Fig. 3, H_2O_2 treatment induced the activation of caspase-9 and -3 significantly, while pretreatment with EG reduced the levels of cleaved caspase-9 and -3 dose-dependently.

Poly (ADP-ribose) polymerase (PARP), a nuclear enzyme involved in DNA repair, is a substrate of caspase-3 during cell death (Boulares et al. 1999). The western blot results also showed that the cleaved PARP decreased as the same trend as caspase-3 (Fig. 3). These results indicated that EG inhibited H_2O_2 induced caspase-9, −3, and PARP activation, thereby suggested EG inhibited H_2O_2-induced caspase-dependent apoptotic process.

EG pretreatment affected the expression of mitochondria-related apoptosis regulatory proteins in PC12 cells

The Bcl-2 family proteins play a fundamental role in the regulation of the intrinsic pathway of apoptosis by regulating permeabilization of the mitochondrial outer membrane and the release of cytochrome *c* (Shi 2001). As aforementioned, EG pretreatment inhibited the decrease of MMP and cytochrome *c* release induced by H_2O_2, here we determined the changes of anti-apoptotic protein Bcl-2, Bcl-xL and pro-apoptotic protein Bax. The western blot results showed that H_2O_2 induced downregulation of Bcl-2 and Bcl-xL, while

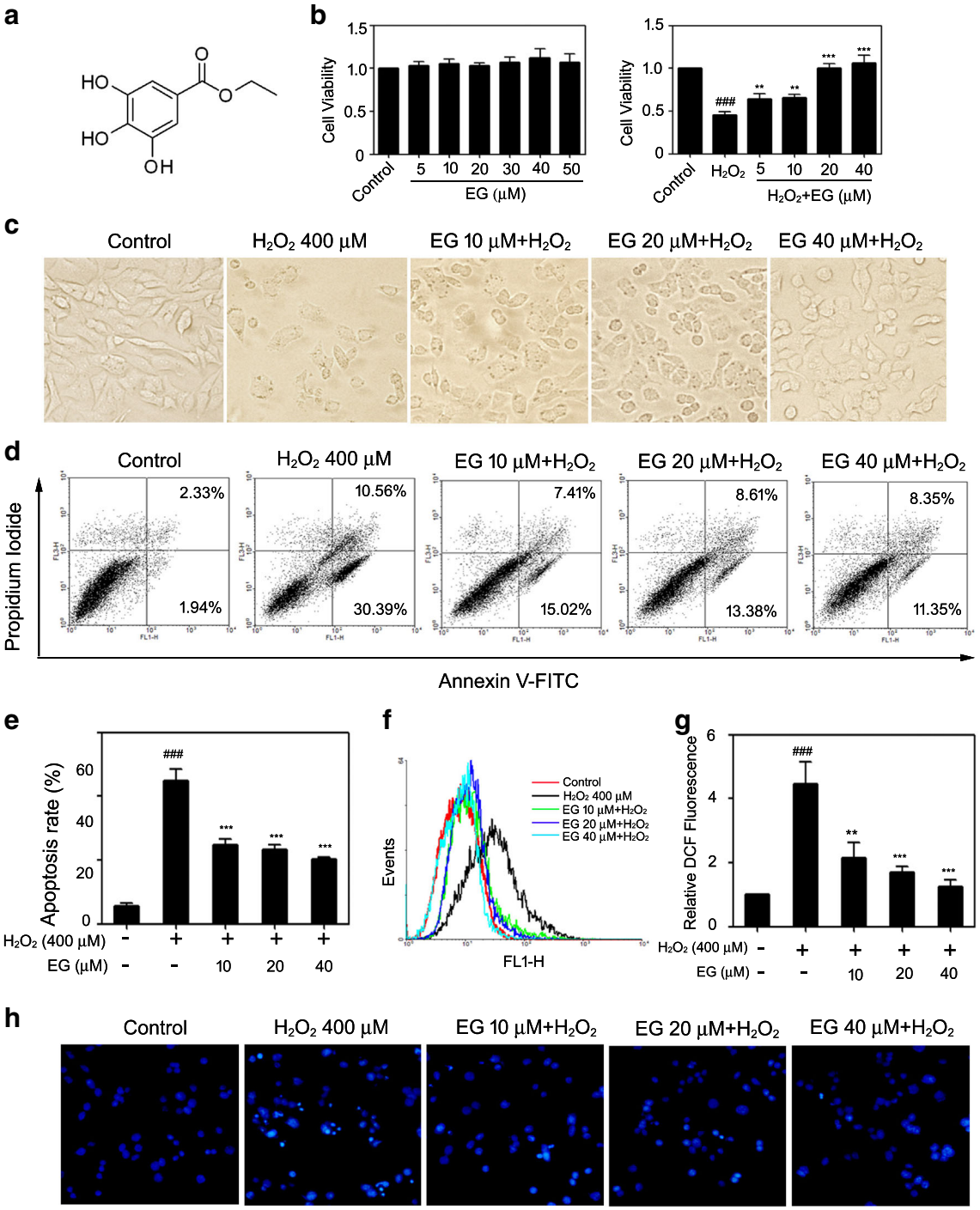

Fig. 1 EG protected PC12 cells against H_2O_2-induced apoptosis and alleviated H_2O_2-induced oxidative stress. **a** The chemical structure of EG. B-E EG protected H_2O_2 induced PC12 cell injury. Cells were incubated with EG alone at indicated concentrations for 24 h (left panel), or pretreated with or without EG (5, 10, 20, 40 μM) for 4 h prior to 400 μM of H_2O_2 exposure for 24 h (right panel). The cell viability was assessed by MTT assay (**b**). Cell morphology was observed under a microscope (**c**). Cellular apoptosis was analyzed with flow cytometry after Annexin V-FITC and PI staining (**d**). Quantification of apoptotic cells were from three independent experiments (**e**). **f** The levels of ROS were detected by flow cytometry with DCFH-DA as fluorescent probe. **g** The relative fluorescence intensity was presented as mean ± SD of three independent experiments. **h** The cells were stained with DAPI and visualized under a fluorescence microscopy. Values are the mean ± SD from three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$ vs. H_2O_2 group and $^{\#\#\#}p < 0.001$ vs. control

Bax was upregulated. EG pretreatment effectively inhibited the expression of Bax and promoted the expression of Bcl-2 and Bcl-xL, indicating EG protected PC12 cells against H_2O_2 induced apoptotic effects (Fig. 4).

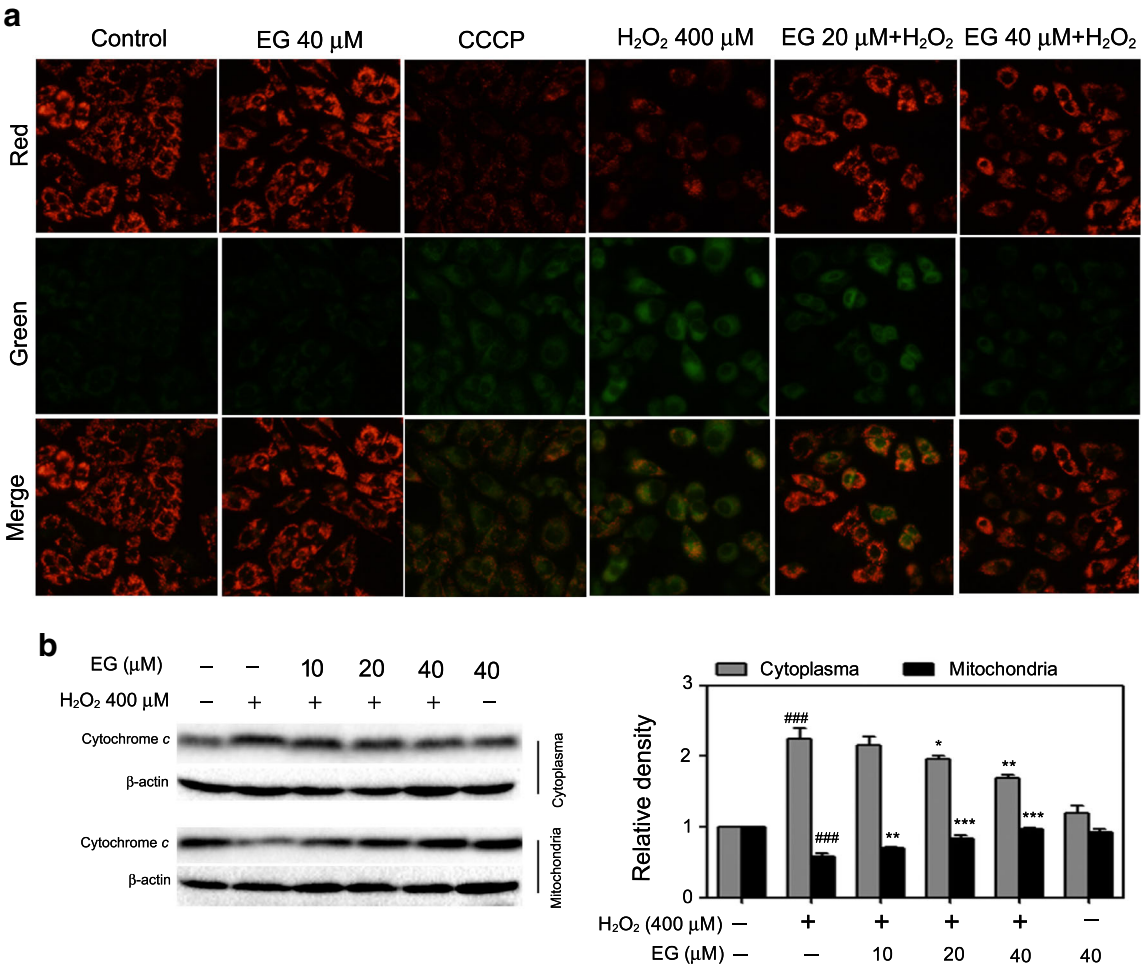

Fig. 2 EG inhibited the decrease of MMP and the release of cytochrome *c* induced by H_2O_2 in PC12 cells (**a**) PC12 cells were incubate with EG for 4 h and then exposed to 400 μM H_2O_2 for 24 h. MMP changes were monitored by loading with JC-1 and detected by fluorescence microscopy. **b** The cytosolic extracts and mitochondrial fraction were analyzed by immunoblotting to detect mitochondrial cytochrome *c* release. β-actin was used as a loading control. The results are mean ± SD from three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. H_2O_2 group and $^{###}p < 0.001$ vs. control

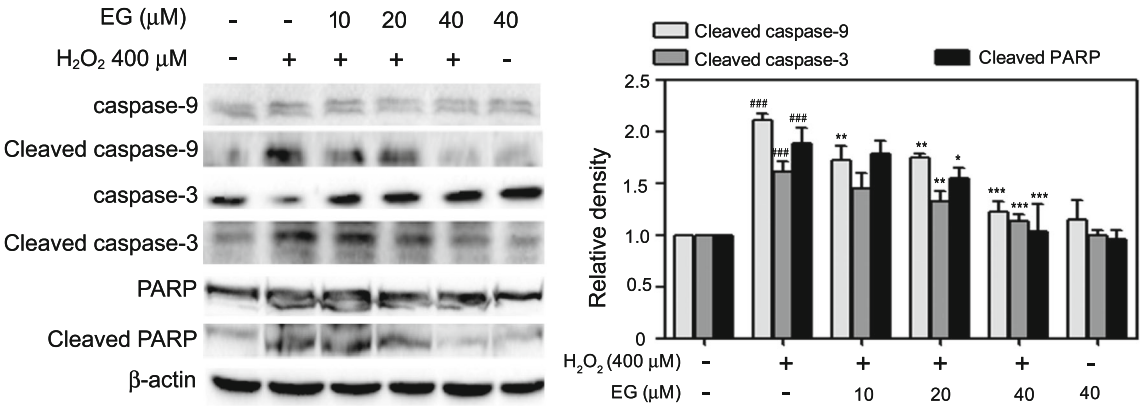

Fig. 3 EG pretreatment inhibited the activation of caspase-9 and caspase-3 induced by H_2O_2 in PC12 cells. Cells were pretreated with or without EG for 4 h prior to 400 μM of H_2O_2 exposure for 24 h, cell lysates were subjected to immunoblotting. The results are mean ± SD from three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. H_2O_2 group and $^{###}p < 0.001$ vs. control

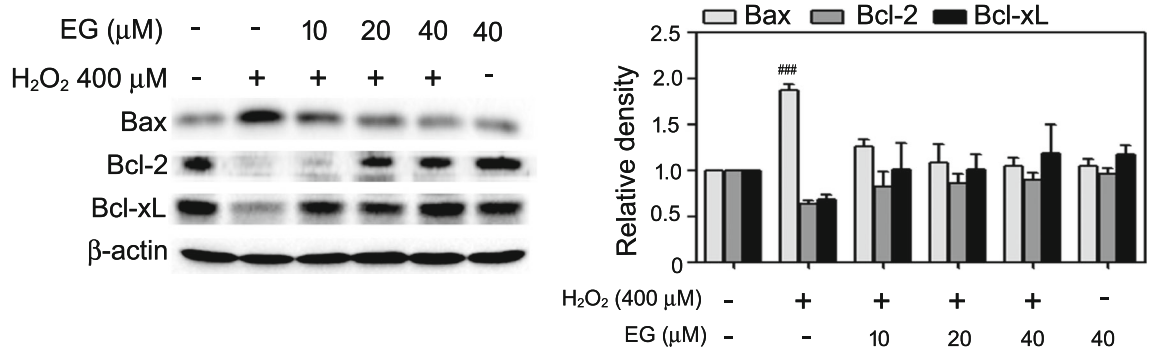

Fig. 4 EG pretreatment affected the expression of mitochondria-related apoptosis regulatory proteins in PC 12 cells. Cells were pretreated with or without EG for 4 h prior to 400 μM of H_2O_2 exposure for 24 h. Cell lysates were subjected to immunoblotting. The results are mean ± SD from three independent experiments. $^{*}p < 0.05$; $^{***}p < 0.001$ vs. H_2O_2 group and $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ vs. control

EG induced Nrf2 nuclear translocation and transcriptional activation in PC12 cells

EG has been reported to exert antioxidant actions, but the underlying mechanisms are not clear yet. Nrf2 signaling pathway is a well-known regulator of cytoprotective responses against oxidative stresses (Baird and Dinkova-Kostova 2011). To gain further insight into the mechanism underlying the protective effects produced by EG against H_2O_2 induced PC12 cells injury, the involvement of EG and Nrf2 pathway was investigated. The classical activation pattern of Nrf2 involves its translocation from cytoplasm into the nucleus. Therefore the first investigation was about whether Nrf2 protein accumulated in nucleus. The results from immunofluorescence showed that Nrf2 was predominantly located in cytoplasm in control group, while after treatment with EG or tBHQ (positive control)(Zhang et al. 2017a), Nrf2 translocated from cytoplasm into nucleus (Fig. 5a). Once Nrf2 translocated in nucleus, the expression of a battery of downstream genes would be activated. As shown in Fig. 5b, the results from western blot analysis revealed the expression of both Nrf2 and its target genes γ-GCS, NQO1 were upregulated after EG treatment. Moreover, the mRNA levels were examined by RT-qPCR. The mRNA expressions of Nrf2, GCLM and NQO1 in EG treatment groups were much higher than those in control group (Fig. 5c). These results suggested that EG treatment could activate Nrf2-mediated endogenous anti-oxidative system in PC12 cells.

Glutathione (GSH) is an important antioxidant that regulates the cellular redox and protects cells from oxidant-induced injury (Schulz et al. 2000). Nrf2 plays a key role in the regulation of cellular GSH homeostasis. After treating PC12 cells by EG for 24 h, the intracellular reduced GSH levels were measured and a dose-dependently increased GSH level was observed (Fig. 5d).

Nrf2 siRNA blocked the cytoprotection of EG in H_2O_2 treated PC12 cells

To access if the activation of the transcription factor Nrf2 is implicated in the protection role of EG, knockdown of Nrf2 was performed. PC12 cells were transiently transfected with Nrf2 siRNA and non-targeting siRNA as a control. As shown in Fig. 6a, Nrf2 siRNA transfection dramatically decreased Nrf2 protein expression, while transfection of the negative control siRNA (NC-siRNA) had no effect on expression of Nrf2. In a cell viability test, cells were transfected with either NC-siRNA or Nrf2 siRNA, treated with DMSO or 40 μM of EG, and then subjected to H_2O_2 challenge. Nrf2 siRNA transfection significantly blocked the cytoprotection of EG, however, in NC-siRNA transfected group, EG still conferred protection on H_2O_2 treated cells (Fig. 6b). We also detected the caspase-3 protein levels, as shown in Fig. 6c, H_2O_2 treatment activated pro-caspase-3 as evidenced by the increased level of cleaved caspase-3. EG treatment abolished H_2O_2-induced activation of caspase-3 in NC-siRNA group, while in Nrf2-siRNA transfected group, the cleaved caspase-3 level was similar with H_2O_2 treatment group, indicating EG treatment could not abrogate the activation of caspase-3 with low Nrf2 protein level. These data suggested that Nrf2 protein was required for EG-dependent cytoprotective effect against H_2O_2.

Discussion

The fruit of *P. emblica* is a good resource of antioxidants, earlier phytochemical studies revealed that tannins, lignans and alkaloids are major constituents of this plant. The dried fruit of *P. emblica* has been used in some traditional medicines and was reported to be beneficial to many kinds of disorders, such as insomnia, dyspepsia, diarrhea, dysentery (Variya et al. 2016) and so on. Recent studies also revealed that the extract of dried fruit of *P. emblica* is relevant to the treatment of Alzheimer's disease and other neurodegenerative diseases. The fruit of *P. emblica* was even considered as a promising gift for the mitigation of Alzheimer's disease (Uddin et al. 2016). In our previous study, we provided evidences on the protective effects of EG, a compound obtained from *P. emblica,* against H_2O_2 induced PC12 cells injury. In the present study, except for confirming

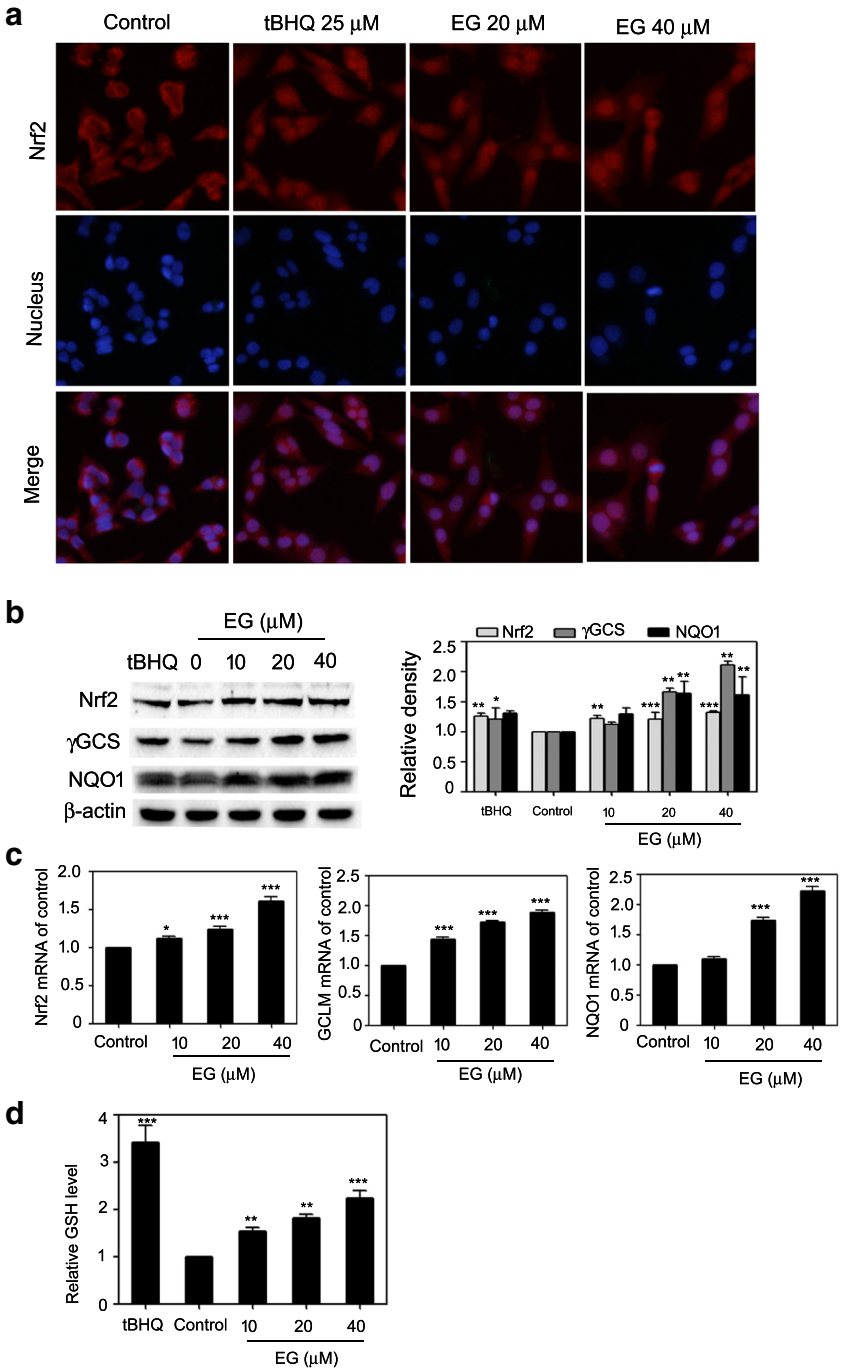

Fig. 5 EG induced Nrf2 nuclear translocation and transcriptional activation in PC12 cells. **a** PC12 cells were treated by the indicated doses of EG and the positive control tBHQ for 4 h, the images were taken by a fluorescence microscope. **b** EG induced the protein expression of Nrf2, NQO1, and γ-GCS in a dose-dependent manner. Total cell lysates from PC12 cells treated with indicated doses of EG for 4 h (Nrf2) and 16 h (NQO1 and γ-GCS) were subjected to immunoblot analysis. **c** EG upregulated the mRNA levels of Nrf2, NQO1 and GCLM. mRNA was extracted from cells treated with indicated doses of EG for 24 h and subjected to qRT-PCR analysis. **d** EG treatment increases GSH levels in a dose-dependent manner. PC12 cells were treated with 10, 20 and 40 μM of EG for 24 h. Cells were harvested for total GSH level analysis. These results are mean ± SD from three independent experiments. $^{*}p < 0.01$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. control

the protective role of EG in reversing H_2O_2-induced damage to PC12 cells, we furthermore elucidated the protective effect was probably correlated with H_2O_2-induced mitochondria dysfunction. Moreover, EG was identified as an Nrf2 pathway inducer, the protective effects of EG was at least partially dependent with its Nrf2 activation effects.

The central nervous system is especially vulnerable to the ROS damage because of its high oxygen demand and its abundance of peroxidation susceptible lipid cells. Oxidative stress has been demonstrated to play a crucial role in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Niedzielska et al. 2016). In general, oxidative stress and mitochondrial dysfunction have been established as elevated in neurodegeneration models. Neurodegenerative diseases are incurable, the progressive degeneration or death of neuron cells, eventually leads to problems with movement or mental functioning. Although it is incurable, the progression of neurodegeneration can be slowed down and the neural cells loss can be limited. Natural antioxidants are considered a promising approach to blunt these processes (Albarracin et al. 2012). Apoptosis is a

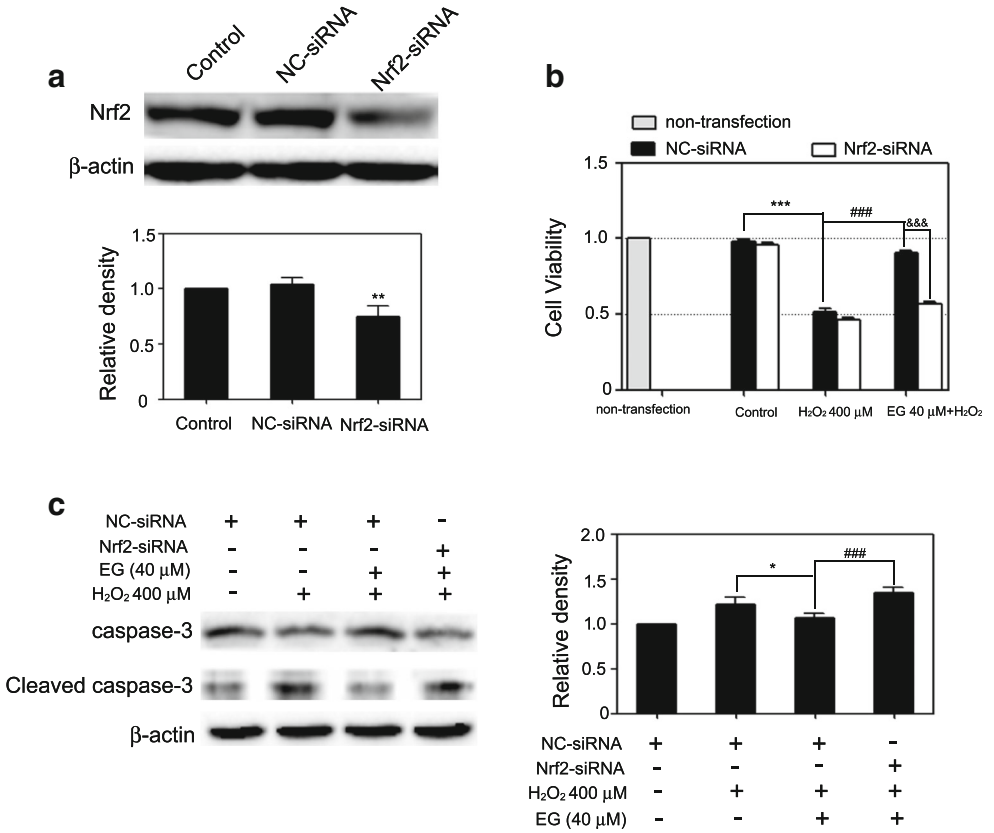

Fig. 6 Nrf2 siRNA blocked the cytoprotection of EG in H_2O_2 treated PC12 cells. **a** Transfection with Nrf2-siRNA silenced the Nrf2 expression. PC12 cells were transfected with control or Nrf2 siRNA for 24 h and cell lysates were subjected to immunoblotting. **b** PC12 cells were transfected with control or Nrf2-siRNA for 24 h, and then pretreated with or without EG for 4 h prior to H_2O_2 exposure for an additional 24 h, the cell viability was measured by MTT assay. **c** EG inhibited the expression of cleaved caspase-3 in an Nrf2-dependent manner. Cells were transfected with control-siRNA or Nrf2-siRNA for 24 h, and treated as indicated manner. Cell lysates were subjected to immunoblot analysis. These values are mean ± SD from three independent experiments. $^{**}p < 0.01$ vs. control; $^{***}p < 0.001$ vs. control; $^{\#\#\#}p < 0.001$ vs. NC-siRNA + H_2O_2 group and $^{\&\&\&}p < 0.001$ vs. NC-siRNA + EG + H_2O_2 group; $^{*}p < 0.05$ vs. NC-siRNA + H_2O_2 group and $^{\#\#\#}p < 0.001$ vs. NC-siRNA + H_2O_2 + EG group

highly regulated and controlled process of cell death during which cells undergoes self-destruction. Apoptosis can be initiated through either intrinsic pathway or extrinsic pathway. Both pathways induce apoptosis by activating initiator caspases first, following cleavage of executioner caspases to dismantle cells (Parrish et al. 2013). In this study, it was demonstrated that pretreatment of PC12 cells with EG suppressed H_2O_2-induced intrinsic apoptosis, which indicated by inhibiting the activation of initiator caspase-9 and effector caspase-3 induced by H_2O_2 treatment. Also EG treatment attenuated the elevation of intracellular ROS level, prevented the depletion of MMP and cytochrome *c* release.

There is growing evidence that low endogenous levels of H_2O_2 act as signaling agent in the regulation of a variety of biological process. However, excess cellular H_2O_2 can be toxic and trigger oxidative stress (Stone and Yang 2006). One feasible way to prevent oxidative stress induced injury and mitochondrial dysfunction may be achieved by stimulation of endogenous cellular mechanisms. The transcription factor Nrf2 is a master regulator of the endogenous antioxidant response (Kensler et al. 2007), which is important in maintaining intracellular redox balance. Nrf2 promotes cell survival by activating the transcription of its downstream targets bearing the antioxidant response element (ARE) in the promoter region. These cytoprotective genes include phase II detoxifying enzymes, intracellular redox-balancing proteins and transporters (Itoh et al. 1997). Based on recent reported data, Nrf2 is regarded as a promising target to combat the oxidative stress in neurodegenerative disorders (Calkins et al. 2009). Thus, we focused on whether the antioxidative effects of EG were related with its regulation of Nrf2 pathway. The results indicated that EG promoted the Nrf2 translocation from cytoplasm into nucleus, increased the protein levels of Nrf2 and its target genes γ-GCS and NQO1, also enhanced the mRNA expressions of Nrf2, GCLM and NQO1. Nrf2-silencing experiments proved that the protection against H_2O_2-induced damage was significantly attenuated. These results suggested that EG activated the Nrf2 pathway, and Nrf2 was indispensable for the protection against H_2O_2-induced oxidative damage in PC12 cells.

Consistant with our findings, a previous report has already shown EG as an Nrf2 activator. In that study, protective effect of EG on LPS-induced mice acute lung injury (ALI) was demonstrated, mechanistic investigation revealed that EG treatment translocated Nrf2 into nuclear both in vivo and in vitro, silencing of Nrf2 through siRNA abrogated the EG induced protective role in LPS induced human monocytes, indicated that EG exert its protective role essentially through Nrf2 signaling (Mehla et al. 2013). However, the current study reported the protective effect of EG in a different system, a H_2O_2-induced oxidative injured PC12 cell model, the protective role of EG in this system is also attributed to the activation of Nrf2 signaling. Although further studies are required to gain a better understanding of the mechanism of action of EG, the current study provide the evidence for the potential value of EG in oxidative stress-induced neuronal cell injury.

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Compliance with ethical standards

Conflict of interest statement The authors declared that there is no conflict of interest associate with this publication.

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