The protective role of (−)-epigallocatechin-3-gallate in thrombin-induced neuronal cell apoptosis and JNK-MAPK activation

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(−)-Epigallocatechin-3-gallate (EGCG), the major polyphenolic component of green tea, has anti-inflammatory and antioxidant properties and provides neuroprotection against central nervous system diseases. Yet, it is not known whether EGCG may be neuroprotective against intracerebral hemorrhage. In this study, we used a simplified in-vitro model of thrombin neurotoxicity to test whether EGCG provides neuroprotection against thrombin-associated toxicity. Exposure of primary cortical neurons to thrombin (100 U/ml) caused dose-dependent and time-dependent cytotoxicity. Cell Counting Kit 8 and lactate dehydrogenase were used to monitor cell viability after exposure of neurons to thrombin or EGCG and after EGCG pretreatment. Flow cytometric analysis and western blotting demonstrated that thrombin-induced neuron degeneration occurs through apoptosis. A concentration of 25 μM EGCG significantly abolished thrombin-induced toxicity and prevented apoptosis by suppressing c-Jun-N-terminal kinase (JNK) phosphorylation, and the JNK inhibitor SP600125 reduced thrombin-induced caspase 3 activation and apoptosis. These data suggest that EGCG may have protective effects against thrombin-induced neuroapoptosis by inhibiting the activation of JNK, leading to caspase 3 cleavage. EGCG is a novel candidate neuroprotective agent against intracerebral hemorrhage-induced neurotoxicity.

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Introduction

Intracerebral hemorrhage (ICH) is a devastating disease that is associated with substantial morbidity and mortality [1]. Poor outcomes following ICH are attributed to a lack of understanding of the underlying mechanisms mediating brain injury after ICH. Neuronal apoptosis plays a key role in the pathophysiological mechanisms underlying ICH, contributing to the development of ICH-induced brain injury [2]. This warrants further investigation of the role of neuronal apoptosis in neurotoxicity following ICH.

(−)-Epigallocatechin-3-gallate (EGCG) is the strongest known antioxidant component in catechins; it exerts multiple protective effects through its antioxidant and antiapoptotic properties [3,4]. EGCG inhibits cell apoptosis through the inhibition of the release of cytochrome *c* into the cytosol, the activation of procaspase 3, and the modulation of cell signaling by activation of the phosphatidyl inositol-3 kinase/Akt pathway and inhibition of GSK-3 [5,6]. Recent studies have shown that EGCG can reduce free radical-induced neuronal apoptosis after a traumatic brain injury [7]. EGCG can also protect neurons from amyloid-β-induced apoptosis [8]. However, it is not clear whether EGCG can protect neurons from thrombin-induced neuronal injury.

In organotypic hippocampal slice cultures, 10 U/ml thrombin induced neuroprotection against experimental 'ischemia' by transient deprivation of oxygen and glucose; however, a higher thrombin concentration (100 U/ml) caused neuronal cell death associated with sustained neuronal Ca^{2+} elevation [9,10]. The progression of pathogenic events following ICH (e.g. cytochrome *c* release, procaspase 3 activation) was prevented by the thrombin inhibitors argatroban and hirudin [11]. c-Jun-N-terminal kinase (JNK) represents one subgroup of the mitogen-activated protein kinase (MAPK) family that plays a critical role in ischemic apoptosis and mitochondrial release of cytochrome *c* [12].

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Evidence suggests that JNK may be a major mediator of apoptotic neuronal damage following ICH. Further, ICH-induced neuron loss was prevented by JNK inhibitors [13].

In the present study, we investigated the possible protective effects of EGCG and the involvement of phospho-JNK in the mechanism of thrombin-induced neuronal cytotoxicity.

Methods

Neuronal cell culture

Primary cortical neuronal cell culture was performed as described previously [14]. Briefly, the cerebral cortical tissues of fetal rats were isolated from pregnant rats, and the isolated cells were seeded at a density of 1×10^6 cells/ml in 25 mm^2 plates (precoated with poly-L-lysine) containing 5 ml of serum-free neurobasal (NB) medium (Gibco, Grand Island, New York, USA) supplemented with 2% B-27 and 12.5% L-glutamine (Sigma, St. Louis, Missouri, USA). Neurons were incubated at 37°C in an incubator with 95% air/5% (v/v) CO_2 (Thermo, Waltham, Massachusetts, USA).

Experiment groups

Neurons were divided into four groups: nontreated group: neurons were treated with NB as a control; thrombin group: neurons were incubated with 100 U/ml thrombin; SP600125 (Beyotime, China) or dimethyl sulfoxide group: neurons were pretreated with 10 μM SP600125 or dimethyl sulfoxide for 2 h and then incubated with 100 U/ml thrombin (T-4648; Sigma); EGCG group: neurons were pretreated with 25 μM EGCG for 24 h and then incubated with 100 U/ml thrombin. All groups were incubated for 10 days in NB medium and treated with 100 U/ml thrombin for 48 h.

Identification of neurons

Neurons were fixed with 4% paraformaldehyde and treated with 0.5% triton permeabilizing agent and 5% donkey serum blocking agent for 30 min. Next, the cells were incubated with mouse anti-β3-tublin antibody (1 : 500; Santa Cruz Biotechnology, Dallas, Texas, USA) at 4°C overnight (1 : 500; Santa Cruz); thereafter, the cells were incubated with rhodamine-conjugated donkey anti-mouse IgG at 37°C for 2 h. Finally, DAPI nuclear counterstain was applied to the cells for 3 min.

Lactate dehydrogenase and Cell Counting Kit 8 assays

Different concentrations of thrombin and cells were cocultured for 24 h. The culture supernatant was collected and added to 96-well plates for lactate dehydrogenase (LDH) determination using the LDH assay kit according to the manufacturer's instructions.

The Cell Counting Kit (Dojindo, Japan) is widely used for assessing cell cytotoxicity. Absorbance is directly proportional to cell proliferation and inversely proportional to cytotoxicity. Cells were divided into three groups: (a) the blank control group, (b) the normal control group, and (c) the experimental group. The blank control group contained the drug and NB medium, the normal control group contained neurons and NB medium, the experimental group contained the drug, neurons, and NB medium. The experimental group was treated as above. Cell viability (%) is calculated according to the following formula: $[(c-a)/(b-a)] \times 100\%$.

The optical density value was measured using a microplate reader at a wavelength of 450 nm. Neuron viability was estimated on the basis of optical density values.

Western blotting

Protein was extracted from neurons, and its concentration was detected using the bicinchoninic acid protein assay kit. The membrane was incubated with primary antibodies from mice or rabbits [anti-P-JNK, anti-JNK (1 : 1000 dilutions; Santa Cruz), anti-caspase 3 (Anbo Biotechnology, San Francisco, California, USA, 1 : 500)] in 5% skimmed milk overnight at 4°C. Thereafter, the membrane was incubated with donkey anti-mouse or anti-rabbit IgG antibodies (1 : 5000, Gibico, Grand Island, New York, USA) with 5% skimmed milk in the dark for 2 h at room temperature. The membranes with antigen–antibody complexes were exposed using an Odyssey instrument (Odyssey: LI-COR Bioscience, Lincoln, Nebraska, USA). Relative densities of the resulting bands were analyzed using Image J program (National Institutes of Health, USA).

Fluorescence-activated cell-sorting analysis

Neurons were cultured in a six-well culture plate (10^5 cells/well) and treated as above. Trypsin (0.125%) was used to digest adherent cells for 5 min and donkey serum (20%; Millipore, Billerica, Massachusetts, USA) was added to terminate digestion. After washing the neurons with PBS, the cell suspension was centrifuged at 1200 rpm for 5 min. Cells were extracted with 500 μl of binding buffer and were incubated in dark for 10 min at room temperature with 5 μl of Annexin V–FITC (20 μg/ml) and 5 μl of propidium iodide (20 μg/ml) working solution [Nanjingkaiji Bioengineering Institute (Kaiji Biotechnology, Nanjing, Jiangsu Province, China)]. The samples were assessed on a flow cytometer and analyzed using CellQuest (BD Biosciences, New Jersey, New York, USA) software.

Statistics analysis

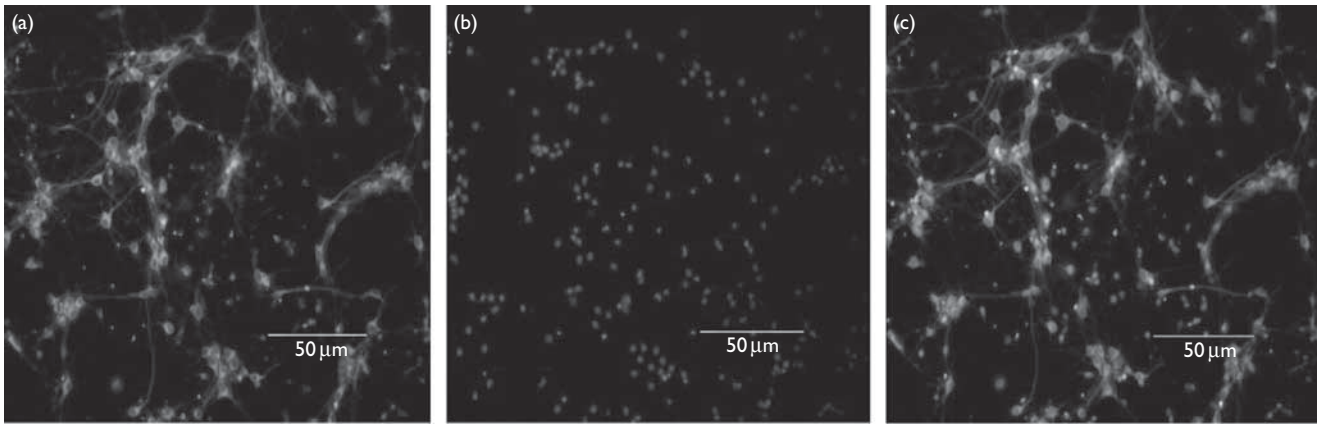
All data were expressed as mean ± SE. Statistical significance was analyzed by one-way analysis of variance, followed by Tukey's multiple comparative test or Dunnett's T3 test. The significance level was determined at P less than 0.05.

Results

Assessment of the neurons' purity

Perikarya and dendrites of rat cortical neurons were labeled with a neuron-specific antibody, β3-tublin, and stained red. The nuclei were stained blue with DAPI. Neurons were seen to comprise roughly 90% of all nucleated cells (Fig. 1).

Fig. 1



Assessment of neuron purity. (a) β3-Tublin-marked neurons showed red fluorescence. (b) Nuclei counterstained with DAPI showed blue fluorescence. (c) Colocalization of cytoplasm β3-tublin and nuclear DAPI (scale bar, 50 μm). DAPI, 4',6-diamidino-2-phenylindole.

Neuronal viability

A dose gradient of thrombin or EGCG incubated with the cells revealed that neuron viability was dose-dependent. Figure 2a shows that the optimal concentration of thrombin for induction was 50 or 100 U/ml. To achieve the best effect, we chose 100 U/ml thrombin for the subsequent experiment. LDH released from neurons treated with 50 μM EGCG was remarkably higher than that released by the control cell group (Fig. 2b). When neurons were pretreated with EGCG for 24 h and then treated with thrombin for 48 h, EGCG inhibited the thrombin-induced decrease in neuron viability (Fig. 2c and d).

c-Jun-N-terminal kinase/caspase 3 activation

The level of JNK phosphorylation increased mildly at 1 h, increased significantly from 3 h to peak at 12 h, and then began to decrease after thrombin exposure. Thrombin-induced activation of caspase 3 showed an early peak at 1 h, followed by a second peak at 24 h.

Compared with the control group, the expression of phospho-JNK and cleaved-caspase 3 in the thrombin-treated group was increased. However, in the SP600125 or EGCG pretreated group, phospho-JNK and cleaved-caspase 3 levels were lower than in nonpretreated neurons (Fig. 3a–c).

Neuron apoptosis

Figure 4 shows that neuron apoptosis was significantly increased in the thrombin-treated group compared with the control group. SP600125 or EGCG pretreatment decreased thrombin-induced neuron apoptosis compared with that among nonpretreated neurons.

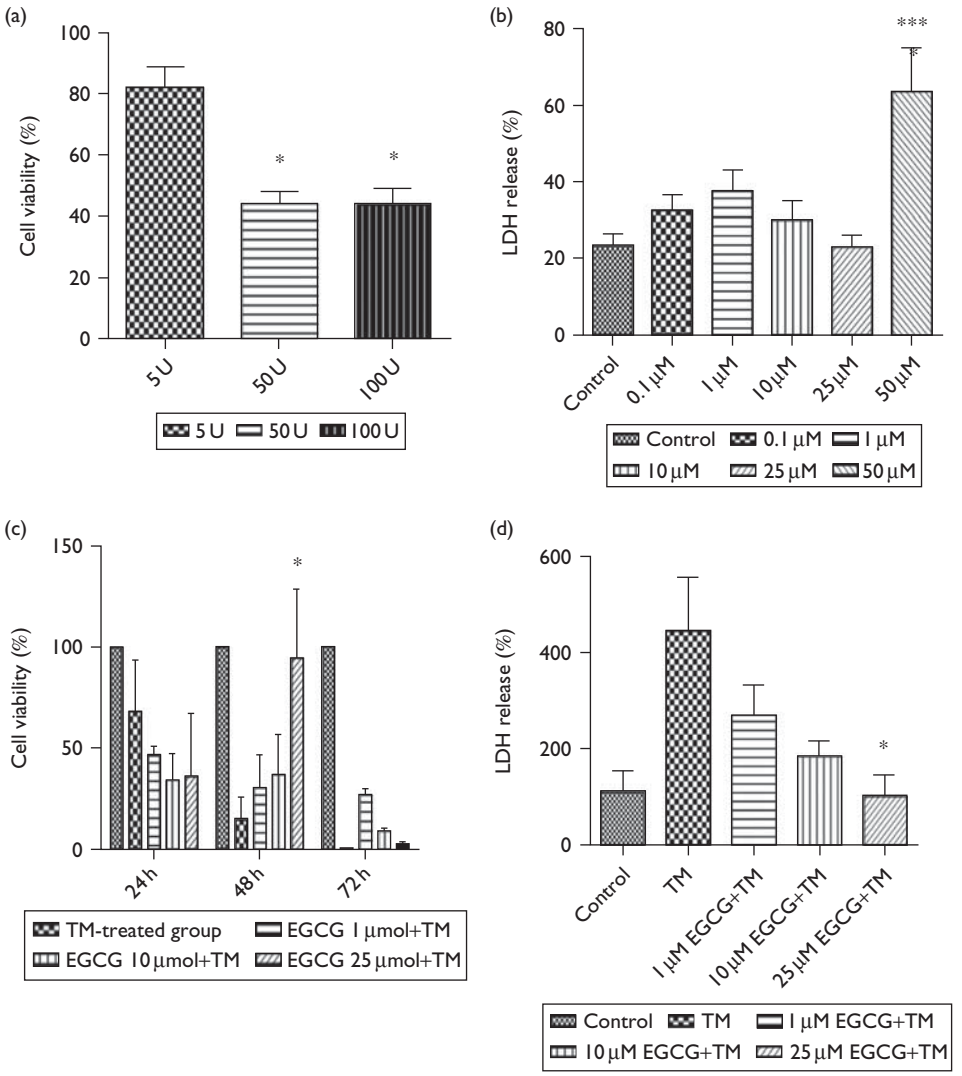
Discussion

In this study, our results reveal involvement of the JNK and caspase 3 pathways in thrombin-induced neuronal apoptosis in primary cortical neurons. Pretreatment of thrombin-treated neurons with 25 μM EGCG significantly decreased neuronal apoptotic activity, as well as the activation of JNK and caspase 3. Therefore, the EGCG-induced decrease in phospho-JNK and caspase 3 activation may contribute toward the antiapoptotic activity against thrombin-induced neuron insults.

MAPKs are cytoplasmic signal transducers that play an important role in thrombin-induced brain tolerance and have ERK, JNK, and p38 MAPK as downstream regulators [7]. Alternate splicing of the three genes *JNK1*, *JNK2*, and *JNK3* generates 10 different JNK isoforms. JNK is implicated in processes such as cellular proliferation, inflammatory response, and cell apoptosis [15]. *JNK3* induces brain injury by transcriptional induction of death-promoting genes and modulation of the mitochondrial apoptosis pathways; targeted deletion of *JNK3* protects mice from brain injury after cerebral ischemia–hypoxia [14]. Moreover, MAPK inhibitors in the ERK, p38 MAPK, and JNK pathways were found to have a partial neuroprotective effect against thrombin-induced shrinkage of the striatal tissue in the organotypic corticostriatal slice in a prior study [16], and treatment with SP600125 (a JNK inhibitor) significantly increased the number of surviving neurons in the peripheral region of the hematoma and at the hematoma center [17]. JNK served as an apoptotic mediator in this article, showing a higher expression level during thrombin-induced neuron injury. Further, SP600125 inhibited caspase 3 activation and thrombin-induced apoptosis.

Thrombin, a serine protease found in the brain after ICH, may be involved in the central secondary mechanism of brain injury in ICH. High doses of thrombin are well-known to be cytotoxic to neurons and astrocytes *in vitro* and to lead to the disruption of the

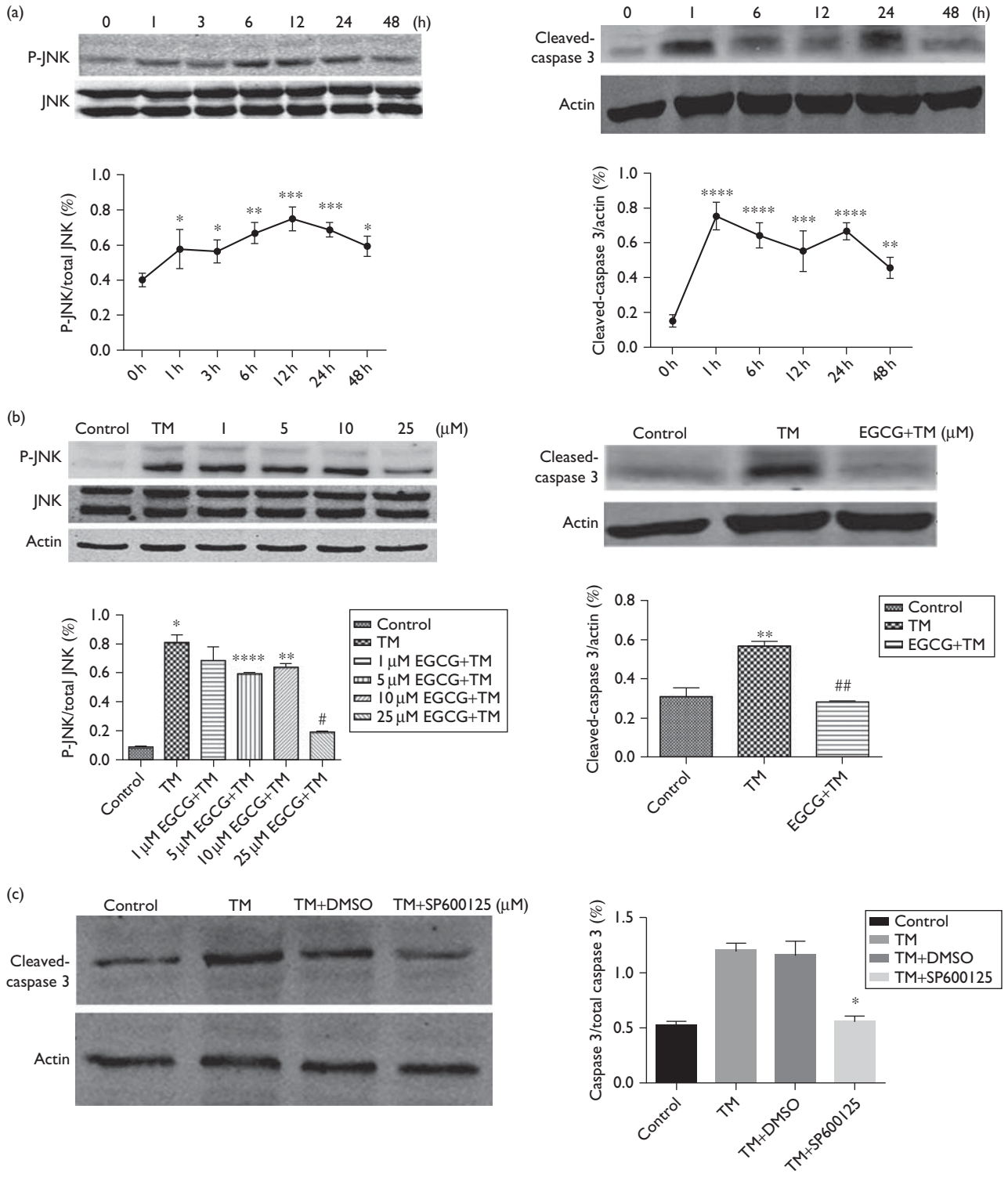
Fig. 2



CCK8 and LDH assays were used to detect cell viability. (a, b) Neuron cell viability after exposure of neurons to thrombin or EGCG with a different concentration gradient (*$P < 0.05$ vs. 5 U/ml thrombin, ***$P < 0.001$ vs. control group). (c, d) Neuron cell damage increased in the thrombin group and EGCG pretreatment significantly increased cell viability (*$P < 0.05$ vs. control group). Columns represent the mean ± SE. $n = 4$. CCK8, Cell Counting Kit 8; EGCG, (−)-epigallocatechin-3-gallate; LDH, lactate dehydrogenase; TM, thrombin.

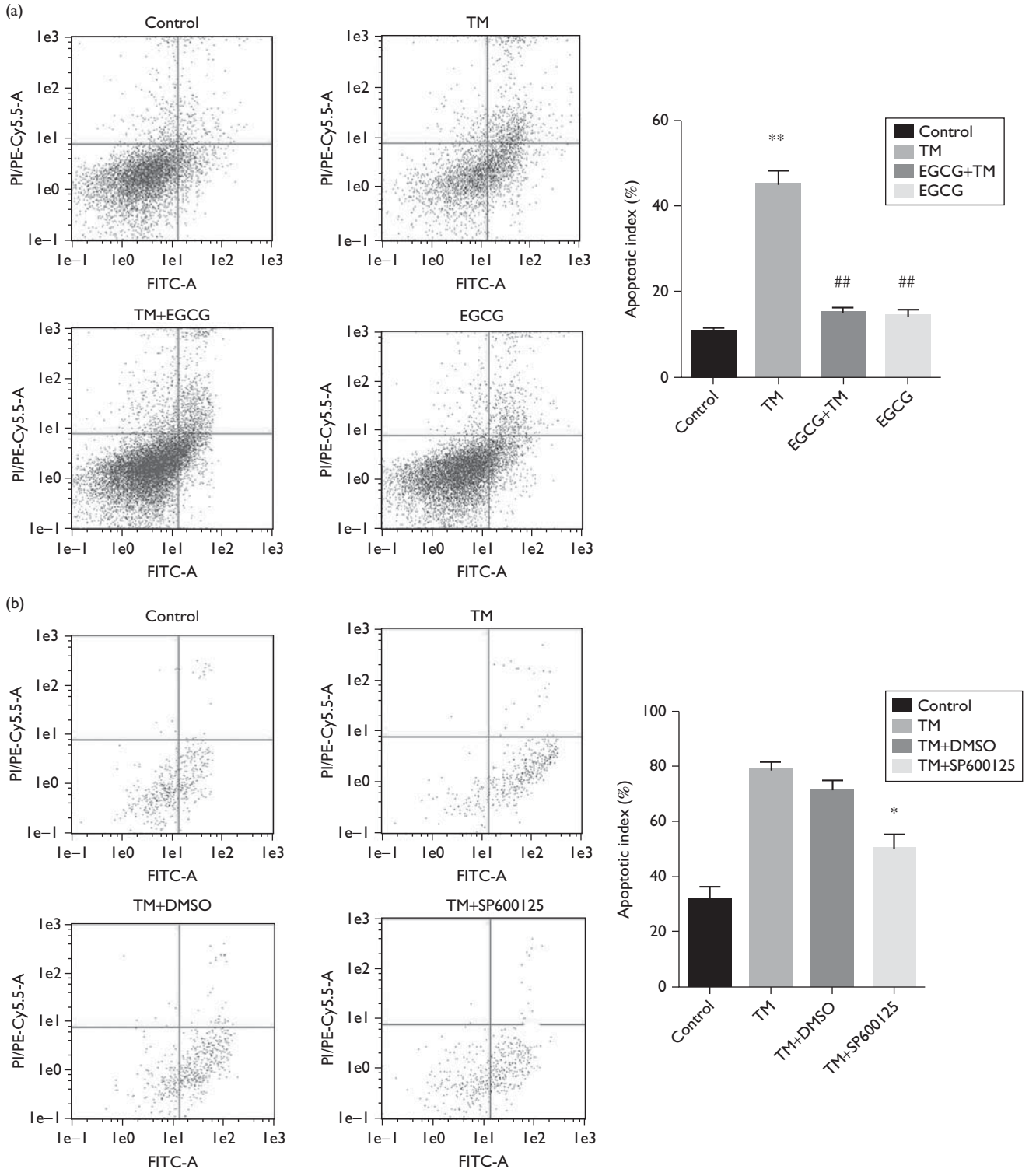
blood–brain barrier and to brain edema *in vivo*. Secondary injury caused by high thrombin concentrations primarily occurs through protease-activated receptors (PARs), a family of G-protein-coupled proteins that exists on the surface of neurons. The thrombin-elicited intracellular responses were mediated by a family of PARs, namely, PAR-1, PAR-3, and PAR-4 [11]. In particular, the activation of PAR-1 may be related to thrombin-induced brain injury and neurogenesis in the hippocampus of rats [18]. Upregulation of PAR-1 is implicated in the potentiation of NMDA receptors, in neurite retraction, and in the activation of MAPKs, leading to inflammation, DNA fragmentation, and cell apoptosis in ICH models [19]. Another report also demonstrated that the PAR-1/Ask1/JNK pathway mediated apoptosis following surgical brain injury [20], suggesting that thrombin triggered the activation of JNK and subsequent apoptotic responses. In this study, our fluorescence-activated cell-sorting results indicate that exaggerated thrombin (100 U/ml) activity plays an important role in neuronal apoptosis, which may be caused by the activation of JNK in thrombin-induced neurons. To explore the causal relationship between JNK activity and neuronal survival, we used western blotting and flow cytometric analysis to examine the role of SP600125 in neuron caspase 3 activation and neuron apoptosis induced by thrombin, and results showed that SP600125 prevented thrombin-induced apoptosis by suppressing caspase 3 activation. Moreover, we focused

Fig. 3



Activation of JNK and caspase 3 in neuron cells. (a) The time dependence of JNK/caspase 3 activation in thrombin-induced neurons (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ vs. control group, $^{\#\#}P < 0.05$ vs. thrombin group). (b) EGCG pretreatment significantly decreased JNK and caspase 3 activation. (c) SP600125 pretreatment significantly decreased caspase 3 activation (*$P < 0.05$ vs. thrombin group). Columns represent the mean ± SE. $n = 3$. DMSO, dimethyl sulfoxide; EGCG, (−)-epigallocatechin-3-gallate; JNK, c-Jun-N-terminal kinase; TM, thrombin.

Fig. 4



Fluorescence activated cell-sorting Annexin V–FITC/propidium iodide (PI) kit was used to detect cell apoptosis. (a, b) EGCG and SP600125 pretreatment significantly decreased cell apoptosis. $^{*}P < 0.05$ versus the thrombin group, $^{**}P < 0.01$ versus control group, $^{\#\#}P < 0.01$ versus thrombin group. Each column represents the mean ± SE. $n = 3$. DMSO, dimethyl sulfoxide; EGCG, (−)-epigallocatechin-3-gallate; TM, thrombin.

on neuroprotection of EGCG against thrombin-associated toxicity. However, the effect of EGCG in other neural injuries potentially induced by toxic factors needs further investigation [21].

EGCG in green tea contains many biologically important polyphenols and exhibits widely beneficial actions, such as inhibition of growth factor signaling pathways and numerous kinases such as JNK, AP-1, and p44/p42 MAPK [5]. The neuroprotective effects against neuronal cell death after ischemia or traumatic brain injury in rats have been illustrated [4]. Numerous studies over the last decade have revealed that EGCG can resist the deleterious effects of oxygen-derived free radicals and chelated iron. Another study showed that EGCG inhibited thrombin-p42/p44 MAPK-induced hepatocellular carcinoma [5]. However, the role of EGCG in thrombin-induced neuronal injury remains unclear. In this study, we found that EGCG can exert protective effects against the neurotoxicity triggered by thrombin in primary cultured cortical neurons.

It has been reported that the number or neurite length of neurons remains unchanged under treatment with EGCG (0.1, 1, or 10 μM) alone in mesencephalic dissociated cultures [22]. However, 400 μM EGCG led to apoptotic cell death through the ASK1, MKK, and JNK/p38 cascades in human leukemia cells [23]. Moreover, EGCG at appropriate concentrations reduced neuronal cell damage induced by ischemic stroke [24]. Consistent with these studies, our experimental findings demonstrate that low concentrations of EGCG fail to cause damage in dissociated cultured neuronal cells, but high concentrations of EGCG (50 μM) reduce cell viability. Therefore, there is a narrow therapeutic window for use of EGCG as a potential neuroprotective agent. To confirm the effective concentration of EGCG, we used western blotting and LDH/Cell Counting Kit 8/ fluorescence-activated cell-sorting to explore the neuroprotective effects of EGCG against thrombin-induced neuronal apoptosis. Finally, we found that pretreatment with 25 μM EGCG inhibits apoptotic response, as evaluated on the basis of the expression of apoptotic mediators such as JNK and caspase 3 in thrombin-induced neurons compared with nonpretreated cells [25]. Taken together, these data indicate that EGCG can curtail the apoptotic response on thrombin-induced neuron insults *in vitro*; the JNK and caspase 3 pathways may play a vital role in the thrombin-induced apoptotic response. However, before this drug is moved into clinical investigation, additional studies are necessary to dissect the explicit pathway involved in the neuroprotection by EGCG against thrombin-induced neuronal apoptosis in ICH.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

References

1 Sangha N, Gonzales NR. Treatment targets in intracerebral hemorrhage. *Neurotherapeutics* 2011; **8**:374–387.
2 Ke K, Song Y, Shen J, Niu M, Zhang H, Yuan D, *et al*. Up-regulation of Glis2 involves in neuronal apoptosis after intracerebral hemorrhage in adult rats. *Cell Mol Neurobiol* 2014.
3 Shimmyo Y, Kihara T, Akaike A, Niidome T, Sugimoto H. Epigallocatechin-3-gallate and curcumin suppress amyloid beta-induced beta-site APP cleaving enzyme-1 upregulation. *Neuroreport* 2008; **19**: 1329–1333.
4 Kaufmann R, Henklein P, Henklein P, Settmacher U. Green tea polyphenol epigallocatechin-3-gallate inhibits thrombin-induced hepatocellular carcinoma cell invasion and p42/p44-MAPKinase activation. *Oncol Rep* 2009; **21**:1261–1267.
5 Adikesavan G, Vinayagam MM, Abdulrahman LA, Chinnasamy T. (−)-Epigallocatechin-gallate (EGCG) stabilize the mitochondrial enzymes and inhibits the apoptosis in cigarette smoke-induced myocardial dysfunction in rats. *Mol Biol Rep* 2013; **40**:6533–6545.
6 Koh SH, Kim SH, Kwon H, Park Y, Kim KS, Song CW, *et al*. Epigallocatechin gallate protects nerve growth factor differentiated PC12 cells from oxidative-radical-stress-induced apoptosis through its effect on phosphoinositide 3-kinase/Akt and glycogen synthase kinase-3. *Brain Res Mol Brain Res* 2003; **118**:72–81.
7 Itoh T, Tabuchi M, Mizuguchi N, Imano M, Tsubaki M, Nishida S, *et al*. Neuroprotective effect of (−)-epigallocatechin-3-gallate in rats when administered pre- or post-traumatic brain injury. *J Neural Transm* 2013; **120**:767–783.
8 Zhang X, Wu M, Lu F, Luo N, He ZP, Yang H. Involvement of alpha7 nAChR signaling cascade in epigallocatechin gallate suppression of beta-amyloid-induced apoptotic cortical neuronal insults. *Mol Neurobiol* 2014; **49**:66–77.
9 Striggow F, Riek M, Breder J, Henrich-Noack P, Reymann KG, Reiser G. The protease thrombin is an endogenous mediator of hippocampal neuroprotection against ischemia at low concentrations but causes degeneration at high concentrations. *Proc Natl Acad Sci USA* 2000; **97**:2264–2269.
10 Xi G, Reiser G, Keep RF. The role of thrombin and thrombin receptors in ischemic, hemorrhagic and traumatic brain injury: deleterious or protective? *J Neurochem* 2003; **84**:3–9.
11 Zhou ZH, Qu F, Zhang CD. Systemic administration of argatroban inhibits protease-activated receptor-1 expression in perihematomal tissue in rats with intracerebral hemorrhage. *Brain Res Bull* 2011; **86**:235–238.
12 Kuan CY, Whitmarsh AJ, Yang DD, Liao G, Schloemer AJ, Dong C, *et al*. A critical role of neural-specific JNK3 for ischemic apoptosis. *Proc Natl Acad Sci USA* 2003; **100**:15184–15189.
13 Wan S, Zhan R, Zheng S, Hua Y, Xi G. Activation of c-Jun-N-terminal kinase in a rat model of intracerebral hemorrhage: the role of iron. *Neurosci Res* 2009; **63**:100–105.
14 Cui G, Wang X, Ye X, Zu J, Zan K, Hua F. Oxygen-glucose deprivation of neurons transfected with toll-like receptor 3-siRNA: Determination of an optimal transfection sequence. *Neural Regen Res* 2013; **8**:3233–3240.
15 Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr Opin Cell Biol* 2007; **19**:142–149.
16 Fujimoto S, Katsuki H, Kume T, Akaike A. Thrombin-induced delayed injury involves multiple and distinct signaling pathways in the cerebral cortex and the striatum in organotypic slice cultures. *Neurobiol Dis* 2006; **22**:130–142.
17 Ohnishi M, Katsuki H, Fujimoto S, Takagi M, Kume T, Akaike A. Involvement of thrombin and mitogen-activated protein kinase pathways in hemorrhagic brain injury. *Exp Neurol* 2007; **206**:43–52.
18 Liu F, Liu F, Wang L, Hu H. Roles of protease-activated receptor-1 in thrombin-induced brain injury and neurogenesis in rats. *Zhejiang Da Xue Xue Bao Yi Xue Ban* 2013; **42**:283–290.
19 Babu R, Bagley JH, Di C, Friedman AH, Adamson C. Thrombin and hemin as central factors in the mechanisms of intracerebral hemorrhage-induced secondary brain injury and as potential targets for intervention. *Neurosurg Focus* 2012; **32**:E8.

20 Manaenko A, Sun X, Kim CH, Yan J, Ma Q, Zhang JH. PAR-1 antagonist SCH79797 ameliorates apoptosis following surgical brain injury through inhibition of ASK1-JNK in rats. *Neurobiol Dis* 2013; **50**:13–20.
21 Xi G, Keep RF, Hoff JT. Mechanisms of brain injury after intracerebral haemorrhage. *Lancet Neurol* 2006; **5**:53–63.
22 Moldzio R, Radad K, Krewenka C, Kranner B, Duvigneau JC, Wang Y, Rausch WD. Effects of epigallocatechin gallate on rotenone-injured murine brain cultures. *J Neural Transm* 2010; **117**:5–12.
23 Saeki K, Kobayashi N, Inazawa Y, Zhang H, Nishitoh H, Ichijo H, *et al*. Oxidation-triggered c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase pathways for apoptosis in human leukaemic cells stimulated by epigallocatechin-3-gallate (EGCG): a distinct pathway from those of chemically induced and receptor-mediated apoptosis. *Biochem J* 2002; **368** (Pt 3):705–720.
24 Park JW, Jang YH, Kim JM, Lee H, Park WK, Lim MB, *et al*. Green tea polyphenol (−)-epigallocatechin gallate reduces neuronal cell damage and up-regulation of MMP-9 activity in hippocampal CA1 and CA2 areas following transient global cerebral ischemia. *J Neurosci Res* 2009; **87**:567–575.
25 Kamalden TA, Ji D, Osborne NN. Rotenone-induced death of RGC-5 cells is caspase independent, involves the JNK and p38 pathways and is attenuated by specific green tea flavonoids. *Neurochem Res* 2012; **37**:1091–1101.