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(−)-Epicatechin, a Natural Flavonoid Compound, Protects Astrocytes Against Hemoglobin Toxicity via Nrf2 and AP-1 Signaling Pathways

Xi Lan[1] · **Xiaoning Han**[1] · **Qian Li**[1] · **Jian Wang**[1]

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Abstract (−)-Epicatechin is a brain-permeable, natural product found at high concentrations in green tea and cocoa. Our previous research has shown that (−)-epicatechin treatment reduces hemorrhagic stroke injury via nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway in vivo. However, the mechanism of action of this compound in modulation of oxidant stress and in protection against hemoglobin-induced astrocyte injury is unclear. Therefore, we explored the cellular and molecular mechanisms that underlie these protective effects in vitro. Mouse primary astrocytes isolated from wild-type mice and Nrf2 knockout (KO) mice were preconditioned with hemoglobin to simulate intracerebral hemorrhage (ICH) in vitro. Effects of (−)-epicatechin were measured by Western blotting, immunostaining, MTT assay, and reactive oxidant stress (ROS) assay. (−)-Epicatechin increased Nrf2 nuclear accumulation and cytoplasmic levels of superoxide dismutase 1 (SOD1) in wild-type astrocytes but did not increase SOD1 expression in Nrf2 knockout (KO) astrocytes. Furthermore, (−)-epicatechin treatment did not alter heme oxygenase 1 (HO1) expression in wild-type astrocytes after hemoglobin exposure, but it did decrease HO1 expression in similarly treated Nrf2 KO astrocytes. In both wild-type and Nrf2 KO astrocytes, (−)-epicatechin suppressed phosphorylated JNK and nuclear expression of JNK, c-jun, and c-fos, indicating that inhibition of activator protein-1 (AP-1) activity by (−)-epicatechin is Nrf2-independent. These novel findings indicate that (−)-epicatechin protects astrocytes against hemoglobin toxicity through upregulation of Nrf2 and inhibition of AP-1 activity. These cellular and molecular effects may partially explain the cerebroprotection as we previously observed for (−)-epicatechin in animal models of ICH.

Keywords (−)-Epicatechin · Heme oxygenase 1 · Intracerebral hemorrhage · Reactive oxygen species · Nuclear factor erythroid 2-related factor 2

Abbreviations

AP-1	Activator protein-1
DAPI	4,6-diamidino-2-phenylindole
EC	(−)-Epicatechin
HO1	Heme oxygenase 1
ICH	Intracerebral hemorrhage
Nrf2	Nuclear factor erythroid 2-related factor 2
ROS	Reactive oxygen species
SOD1	Superoxide dismutase 1

Introduction

Intracerebral hemorrhage (ICH) is a severe subtype of stroke that affects more than 1 million people worldwide annually and accounts for 10–30% of all strokes [1]. Although it is associated with high morbidity and mortality throughout the world, effective therapies are limited. The primary injury after ICH is caused by

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✉ Jian Wang
jwang79@jhmi.edu

[1] Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins University School of Medicine, 720 Rutland Ave, Ross Bldg 370B, Baltimore, MD 21205, USA

bleeding. In particular, hemoglobin released from damaged erythrocytes in the hematoma triggers oxidant stress, neuroinflammation, and neurotoxicity that contribute to secondary brain damage [2–4].

Oxidative stress caused by reactive oxygen species (ROS) plays an important role in ICH progression. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates the expression of antioxidant response element (ARE)-related genes and mitigates secondary brain injury after ICH [3–5]. Using Nrf2 knockout (KO) mice and Nrf2 inducers, we and others have demonstrated that Nrf2 has neuroprotective effects in animal models of ICH [6–9]. Moreover, astrocytic activation of Nrf2 and the genes it regulates downstream, including heme oxygenase 1 (HO1), superoxide dismutase 1 (SOD1), and NAD(P)H oxidoreductase 1 (NQO1), can protect neurons from cytotoxicity both in vitro and in vivo [10–13]. These findings suggest that astrocytic Nrf2 activation is important after ICH.

The activator protein-1 (AP-1) is another transcription factor shown to be involved in regulation of inflammation, apoptosis, cell proliferation/differentiation, and oxidative stress [14]. The AP-1 complex is composed of proteins from the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) families [15]. Studies have shown that AP-1 DNA binding activity is elevated in in vitro and in vivo models of cerebral ischemia [16–18]; thus, it might contribute to secondary brain injury [14, 19]. One study showed that the Jun amino-terminal kinase (JNK)/c-jun/AP-1 pathway is activated in astrocytes under conditions of oxygen-glucose deprivation [18]. However, the role of AP-1 in ICH is unclear.

(−)-Epicatechin (EC) is a brain-permeable, natural flavanol compound extracted from green tea and cocoa [20, 21] that exerts protective effects on cognition [22], vascular function [23], and ischemic stroke injury [24] in animals and humans. EC has the ability to cross the blood-brain barrier (BBB) both in vivo [25] and in vitro [26]. Furthermore, we have shown that EC ameliorates histologic and functional deficits after ICH and traumatic brain injury. Its effects are associated with a decrease in HO1 expression and increases in SOD1 and NQO1 expression, possibly via activation of Nrf2-dependent and Nrf2-independent pathways [7, 27]. All of these results indicate that EC could provide a promising treatment for ICH. However, the cell-specific function of EC remains largely unknown, and whether EC regulates Nrf2/HO1 in astrocytes needs to be determined.

To further explore the cellular and molecular mechanisms by which EC protects against ICH injury, in this study, we cultured primary astrocytes isolated from wild-type (WT) and Nrf2 KO mice and exposed them to hemoglobin to simulate ICH in vitro. Our results indicate that after hemoglobin exposure, AP-1 could be another target of EC in astrocytes in addition to Nrf2. Activation of AP-1 and Nrf2 in astrocytes may partially explain the cerebroprotective effects of EC as we previously observed in animal models of ICH.

Methods

Materials

Compound (−)-epicatechin, hemoglobin, MTT, and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma Aldrich (Allentown, PA, USA). D-Hanks and 0.25% trypsin were purchased from Quality Biological (Gaithersburg, MD, USA). DMEM/F12, FBS, and Alexa Fluor 488 goat anti-rabbit secondary antibody were obtained from Life Technologies (Carlsbad, CA, USA). Antibodies of Nrf2 (H-300), Lamin a/c, and β-actin were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). SOD1 antibody was purchased from Abcam (Cambridge, UK). HO1 antibody was purchased from Stressgen, (San Diego, CA, USA). Antibodies of phospho-SAPK/JNK, SAPK/JNK, c-jun, c-fos, and peroxidase-coupled goat anti-rabbit or anti-mouse secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA).

Mouse Primary Astrocyte Culture

All the experimental procedures were conducted with accordance with guidelines of the National Institutes for Health and approved by the institutional Animal Care and Use committee at Johns Hopkins University School of Medicine. Nrf2 KO mice (on a C57BL/6 background) were originally generated by Dr. Masayuki Yamamoto (Tohoku University, Japan). Primary astrocytes were cultured from newborn WT and Nrf2 KO mice (postnatal day 1) as described previously [28–30]. In brief, cortices were dissected from brain and cut into 1–3 mm^2 pieces. Cells were dissociated in D-Hanks' solution containing 0.25% trypsin and plated onto 75 cm^2 cell culture flasks at 1×10^7 cells/mL in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12) with 10% fetal bovine serum (FBS). After 2 weeks, microglia and oligodendrocytes were removed from the mixed glial cells by shaking at 200 rpm overnight at 37 °C, and astrocytes were cultured in complete DMEM/F12 medium. Astrocytes that were >95% positive for GFAP staining (supplementary Fig. 1) and within five passages were used in our experiments.

(−)-Epicatechin Treatment

Cells were stimulated with 10 μM hemoglobin, and doses of EC were dissolved in 0.1% DMSO. For evaluation of cell signaling and transcription factors (Nrf2, phosphorylated JNK, c-jun, and c-fos), we pretreated cells with EC 1 h before hemoglobin injury and collected samples after 2 h of hemoglobin exposure. For other experiments, cells were preconditioned with hemoglobin beginning 1 h before the addition of EC, and cell supernatant or proteins were collected 24 h later. Astrocytes from WT mice (WT astrocytes) and $Nrf2^{-/-}$ mice (KO astrocytes) were grouped as follows: (1) control: cells were incubated in 0.1% DMSO, which did not cause cell death compared with untreated cells (supplementary Fig. 2); (2) Hb: cells were treated with 10 μM hemoglobin; (3) EC: cells were treated with EC before or after 10 μM hemoglobin stimulation.

MTT Assay

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, thiazolyl blue (MTT), was used to measure astrocyte cytotoxicity and viability. Cells were incubated with MTT (0.5 mg/mL) at 37 °C for 4 h. After the medium was removed, DMSO was added to each well. Absorbance at 550 nm was read on a SpectraMax M2 microplate reader (Molecular Devices, Downingtown, PA, USA).

ROS Measurement

The peroxide-sensitive fluorescent probe DCF-DA was used to detect the production of intracellular ROS [31]. Fluorescence intensity was read on a SpectraMax M2 microplate reader.

Western Blotting

For Western blotting [32], we used antibodies to the following proteins: Nrf2 (1:200), SOD1 (1:2000), HO1 (1:2000), phospho-SAPK/JNK (1:1000), SAPK/JNK (1:1000), Lamin a/c (1:1000), and β-actin (1:4000). Briefly, 30 μg of protein was separated by 4–20% SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% milk or 5% bovine serum albumin, incubated with primary antibodies at 4 °C overnight, and then incubated with peroxidase-coupled goat anti-rabbit or anti-mouse secondary antibody (1:5000). Membranes were immersed in enhanced chemiluminescence (ECL) solution and exposed under an ImageQuant ECL Imager (GE Healthcare, Little Chalfont, UK).

Immunostaining

Mouse primary astrocytes were fixed with 4% paraformaldehyde, blocked with 5% bovine serum albumin, and incubated with primary antibody to c-jun (1:400) or c-fos (1:200) at 4 °C overnight. Cells were then incubated at room temperature with appropriate secondary antibody (Alexa Fluor 488 goat anti-rabbit, 1:1000). Cell nuclei were stained with DAPI. Cell immunofluorescence intensity was calculated with ImageJ software [33], and the measurements followed published protocol [34].

Statistical Analysis

All data are presented as means ± SD of five independent experiments. Comparisons of two groups were analyzed by unpaired, two-tailed t test. Comparisons among multiple groups were calculated by one-way ANOVA or two-way ANOVA multiple comparison with Bonferroni post hoc test. A $p < 0.05$ was considered significant. Nonlinear logistic regression was used to fit concentration-response curves and to calculate IC50.

Results

EC Reduces ROS Production in Hemoglobin-Stimulated Astrocytes via an Nrf2-Independent Pathway

First, we used WT and Nrf2 KO astrocytes to study the effects of EC on ROS. EC cytotoxicity testing showed that concentrations as high as 500 μM EC were not toxic to cells (Fig. 1a) after a 24-h incubation. To study the effects of EC on hemoglobin-exposed astrocytes, we preconditioned the cells with hemoglobin (20 or 10 μM) for 1 h and then added various concentrations of EC for 24 h. Hemoglobin at 20 μM caused 23.4 ± 7.16% cell death in WT astrocytes and 36.3 ± 6.71% cell death in Nrf2 KO astrocytes. However, 20 μM EC significantly increased the viability of both WT and Nrf2 KO astrocytes exposed to 20 μM hemoglobin (Fig. 1b). To avoid the influence of hemoglobin-induced cytotoxicity, we tested lower concentrations of hemoglobin. When astrocytes were exposed to a lower, 10 μM concentration of hemoglobin, which produced little toxicity (Fig. 1c), ROS production in Nrf2 KO astrocytes was almost twice that in WT astrocytes (Fig. 1d). This finding implies that Nrf2 has a critical antioxidant effect in astrocytes. However, 20–500 μM EC decreased ROS production to a similar extent in WT and Nrf2 KO astrocytes (Fig. 1d). The IC50 of EC for ROS inhibition was 54.5 μM in WT astrocytes and 44.1 μM in Nrf2 KO astrocytes (Fig. 1e, 75% inhibition of ROS production, IC75 = 95.02 μM of WT, and 100.25 μM of KO). These results indicate that EC reduces hemoglobin-induced ROS elevation via an Nrf2-independent

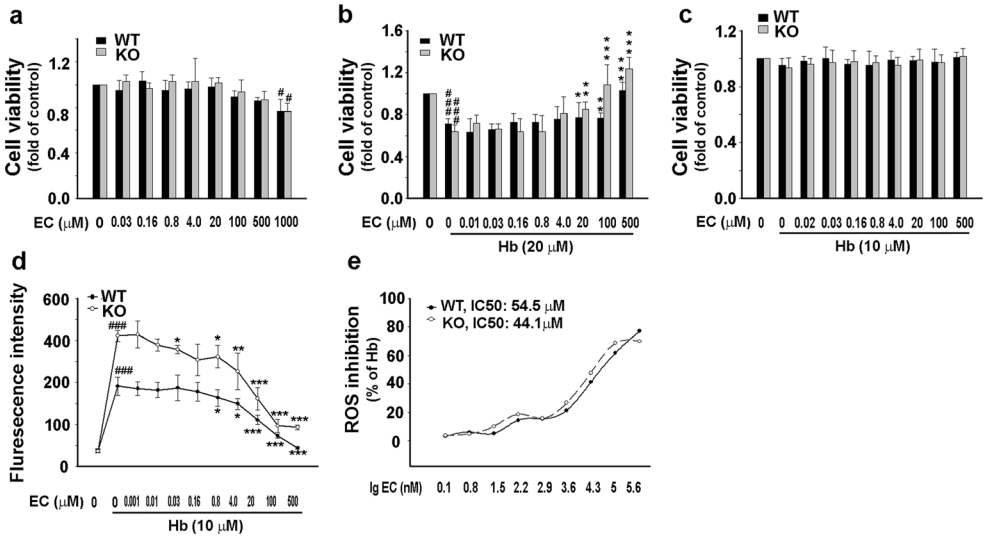

Fig. 1 (−)-Epicatechin reduces reactive oxygen species (ROS) production in hemoglobin-exposed WT and Nrf2 KO astrocytes. **a** WT and Nrf2 KO astrocytes were treated with EC at concentrations from 0.03 to 1000 μM for 48 h. Cytotoxicity was observed only at 1000 μM. **b** Hemoglobin (Hb) at 20 μM caused approximately 23% cell death in WT astrocytes and 36% cell death in Nrf2 KO astrocytes. EC significantly increased cell viability of astrocytes exposed to hemoglobin (20 μM) for 24 h. **c** At a concentration of 10 μM, hemoglobin did not cause cell death in primary astrocytes from WT and Nrf2 KO mice. **d** Hemoglobin-induced ROS production in Nrf2 KO astrocytes was almost twice that in WT astrocytes. EC at concentrations from 0.8 to 500 μM reduced ROS production in astrocytes from Nrf2 KO and WT mice exposed to 10 μM hemoglobin. **e** The IC50 of EC for ROS inhibition was 54.5 μM in WT astrocytes and 44.1 μM in Nrf2 KO astrocytes. All data are presented as mean ± SD of five independent experiments. ###$p < 0.001$ vs. control group; +++$p < 0.001$ vs. WT Hb group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Hb alone

pathway. Based on these data, we selected 100 μM EC for most of the remaining experiments.

EC Increases Nrf2 Expression in Hemoglobin-Stimulated WT Astrocytes

Next, we examined the way EC regulates Nrf2 in astrocytes. For the time point of 2 h, EC was pretreated 1 h prior hemoglobin (10 μM) stimulation. After 2 h, cytoplasmic and nuclear proteins were extracted, and our results showed that nuclear Nrf2 was significantly increased compared with that in the control cells (Fig. 2b). EC (100 μM) further promoted Nrf2 expression in both cytoplasmic (Fig. 2a) and nuclear fractions (Fig. 2b). For the time point of 24 h, EC was added 1 h after hemoglobin exposure, and proteins were extracted 24 h later. Western blotting results showed that cytoplasmic Nrf2 (Fig. 2c) and nuclear Nrf2 (Fig. 2d) remained elevated in EC-treated astrocytes.

Effects of EC on Hemoglobin-Induced HO1 and SOD1 Expression

HO1 and SOD1 are both downstream proteins of Nrf2 translocation and are triggered under oxidant stress. After 24 h of hemoglobin exposure, HO1 expression, but not SOD1 expression, was significantly increased. EC (1, 10, and 100 μM) significantly increased SOD1 expression in WT astrocytes (Fig. 3a) but did not further increase HO1 protein level (Fig. 3a). In contrast, under the same conditions, EC (100 μM) decreased HO1 level in Nrf2 KO astrocytes (Fig. 3b) but had no effect on SOD1 (Fig. 3b). We then measured HO1 and SOD1 levels at 2 h post-hemoglobin. EC (100 μM) did not alter HO1 level in WT astrocytes (Fig. 3c) or Nrf2 KO astrocytes (Fig. 3d). Moreover, EC (100 μM) led to SOD1 upregulation in WT astrocytes but had no effect in Nrf2 KO astrocytes. These data indicate that in hemoglobin-stimulated astrocytes, EC increases SOD1 expression in an Nrf2-dependent manner but regulates HO1 expression in an Nrf2-independent manner.

EC Suppresses the AP-1 Signaling Pathway in both WT and Nrf2 KO Astrocytes

To continue exploring the mechanism of EC in modulation of HO1 and identify the Nrf2-independent targets of EC, we further evaluated AP-1 activation in WT and Nrf2 KO astrocytes. Astrocytes were pretreated with EC (100 μM) for 1 h before being exposed to hemoglobin (10 μM) for 2 h. Then, samples were collected for analysis of JNK/c-jun/AP-1 and c-fos/AP-1. Western blotting showed that in hemoglobin-stimulated WT and KO astrocytes, EC markedly decreased the phosphorylation of JNK (Fig. 4a, c). Immunostaining showed that 100 μM EC prevented JNK nuclear translocation (Fig. 4b, d) and inhibited c-jun nuclear expression (Fig. 4e $p < 0.05$). The nuclear expression of another important AP-1

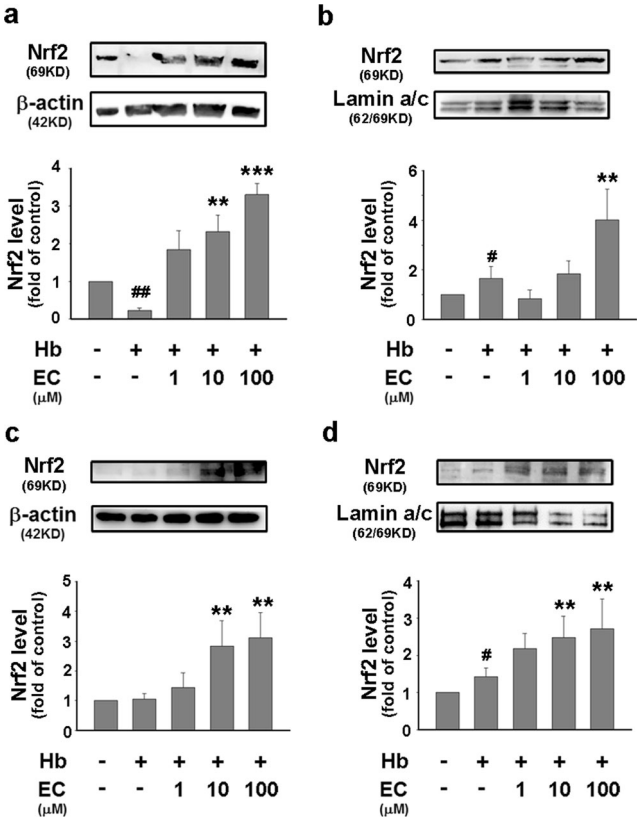

Fig. 2 (−)-Epicatechin increases Nrf2 nuclear translocation in hemoglobin-exposed astrocytes. **a**, **b** EC was added to the medium 1 h before hemoglobin (Hb, 10 μM) treatment, and cytoplasmic (**a**) and nuclear (**b**) protein was extracted 2 h after hemoglobin stimulation. **c**, **d** EC (1, 10, or 100 μM) was added to the medium 1 h after hemoglobin, and cytoplasmic (**c**) and nuclear (**d**) protein was collected 24 h later. All data are presented as mean ± SD of five independent experiments. #$p < 0.05$, ##$p < 0.01$ vs. control group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Hb alone

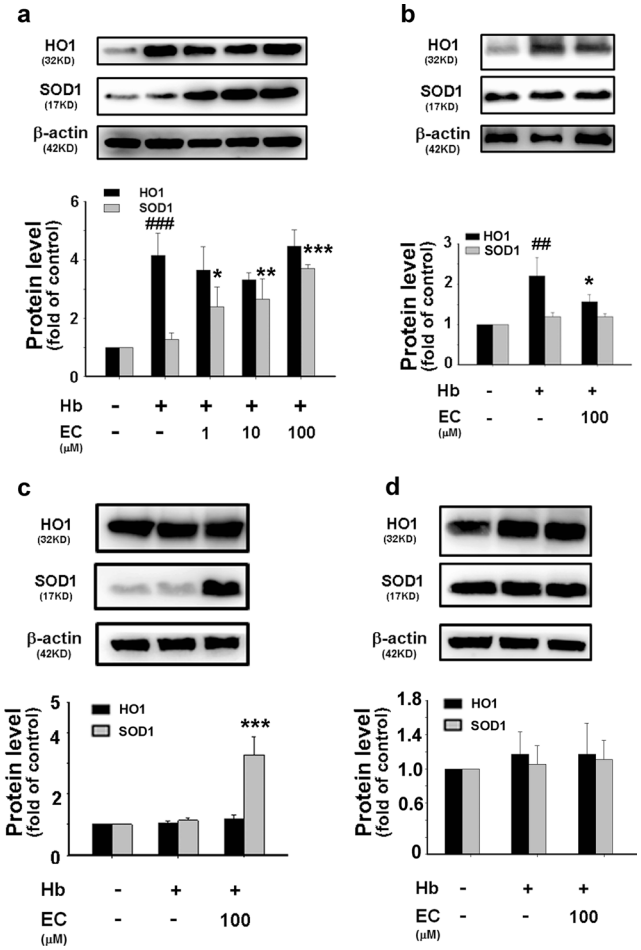

Fig. 3 Effects of (−)-epicatechin on HO1 and SOD1 expression in hemoglobin-exposed astrocytes. **a**, **b** EC was added to the medium 1 h after hemoglobin (Hb, 10 μM). **a** After 24 h, SOD1 expression was significantly increased in WT astrocytes, but HO1 expression was unchanged. **b** At the same time point, 100 μM EC decreased HO1 expression in Nrf2 KO astrocytes without changing SOD1 expression. **c**, **d** EC was added to the medium 1 h before hemoglobin, and protein was collected 2 h after the addition of hemoglobin. **c** EC significantly elevated SOD1 expression in WT astrocytes exposed to hemoglobin for 2 h. **d** EC did not change HO1 or SOD1 expression in Nrf2 KO astrocytes exposed to hemoglobin for 2 h. All data are presented as mean ± SD of five independent experiments. ##$p < 0.01$, ###$p < 0.001$ vs. control group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Hb alone

family protein, c-fos, was also decreased in WT and KO astrocytes by EC treatment ($p < 0.05$, Fig. 4f). These results suggest that AP-1 is another major target of EC for rescuing hemoglobin-damaged astrocytes.

Discussion

In our study, we used hemoglobin-stimulated astrocytes in vitro to investigate the effects of EC, a brain-permeable, natural flavonoid compound with potentially multiple targets. We have shown that EC treatment is protective in animal models of ICH [7] and traumatic brain injury [27]. Here, we further determined the effects of EC on cultured astrocytes exposed to hemoglobin in vitro. We demonstrated for the first time that (1) the IC50 of EC for ROS inhibition is 54.5 μM in WT astrocytes and 44.1 μM in Nrf2 KO astrocytes; (2) EC increases Nrf2 nuclear translocation and expression of its downstream target SOD1 while protecting astrocytes against hemoglobin-induced oxidative injury; (3) the EC-induced increase in SOD1 expression, but not HO1 expression, is lost in Nrf2 KO astrocytes; and (4) hemoglobin increases JNK phosphorylation and nuclear expression of JNK, c-jun, and c-fos in WT and Nrf2 KO astrocytes, and EC inhibits these effects in both genotypes. Together, these findings provide clear in vitro evidence that EC protects astrocytes against hemoglobin toxicity through upregulation of Nrf2 and inhibition of AP-1 activity. Indeed, our finding that EC did not alter HO1 expression in WT astrocytes after hemoglobin exposure may result from a balance between the signaling pathways of Nrf2 activation and AP-1 inhibition (Fig. 5).

Nrf2 could be a therapeutic target for ICH. We and others have shown that Nrf2 deletion exacerbates ICH-induced DNA

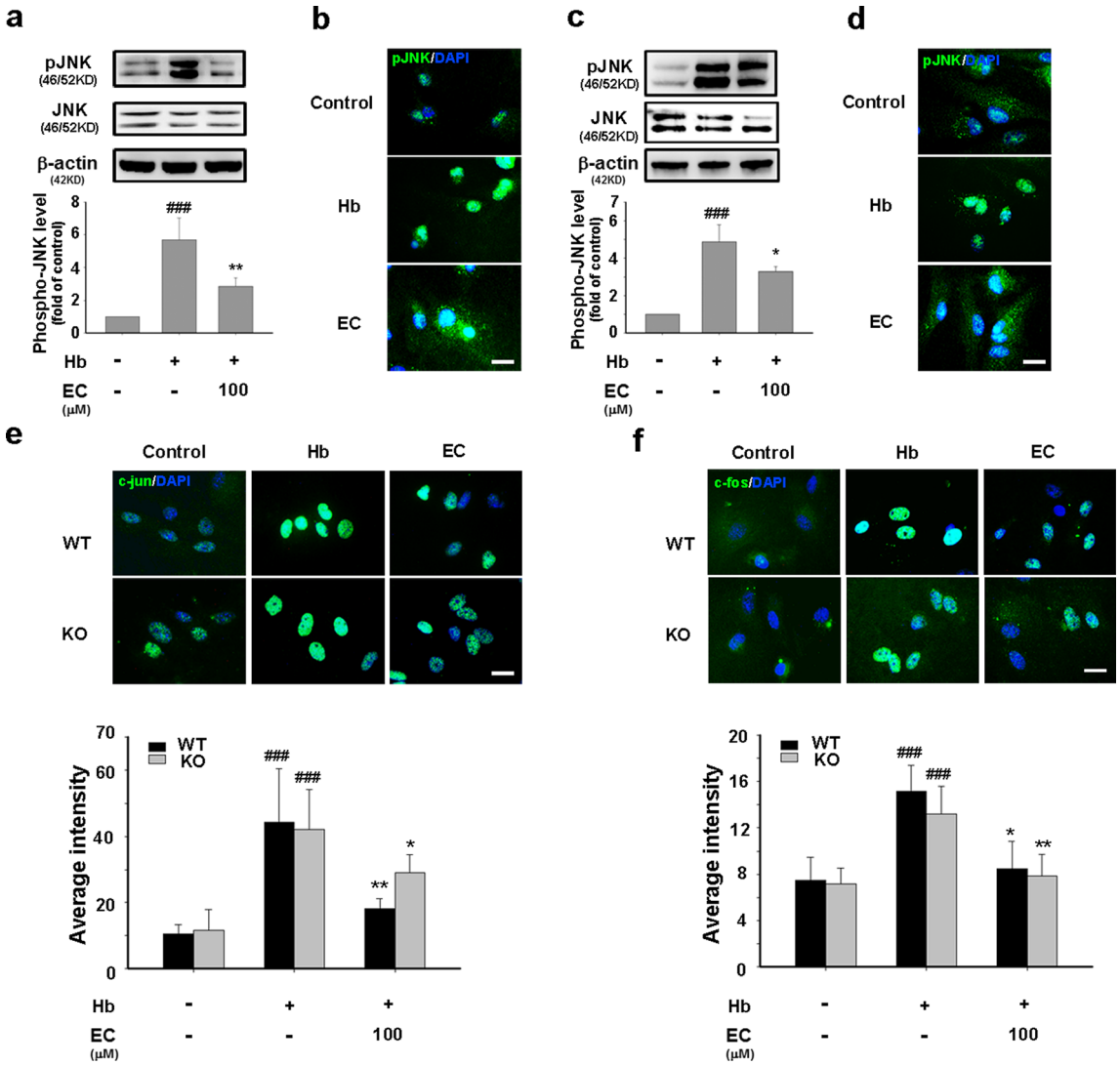

Fig. 4 (−)-Epicatechin suppresses AP-1 signaling in both WT and Nrf2 KO astrocytes. Astrocytes were pretreated with 100 μM EC for 1 h before being exposed to hemoglobin (Hb, 10 μM) for 2 h. **a** EC inhibited the phosphorylation of JNK in WT astrocytes exposed to hemoglobin. **b** Immunostaining showed that EC inhibited phosphorylated-JNK (pJNK, *green*) translocation (DAPI, *blue*) in WT astrocytes exposed to hemoglobin. **c** EC inhibited the phosphorylation of JNK in Nrf2 KO astrocytes exposed to hemoglobin. **d** Immunostaining showed that EC inhibited pJNK (*green*) translocation (DAPI, *blue*) in Nrf2 KO astrocytes exposed to hemoglobin. **e** Immunostaining showed that EC inhibited nuclear expression (DAPI, *blue*) of c-jun (*green*) in WT and Nrf2 KO astrocytes exposed to hemoglobin. **f** Immunostaining showed that EC reduced nuclear (DAPI, *blue*) expression of c-fos (*green*) in WT and Nrf2 KO astrocytes exposed to hemoglobin. All data are presented as mean ± SD of five independent experiments. Scale bar = 20 μm. ###$p < 0.001$ vs. control group; *$p < 0.05$, **$p < 0.01$ vs. Hb alone

damage and apoptosis in neurons [6], whereas Nrf2 activation mitigates ICH injury [7, 8, 35]. Nrf2 can be induced in microglia [36]. It also upregulates scavenger receptor CD36 expression and enhances the microglial phagocytosis of red blood cells [37]. After transient cerebral ischemia, Nrf2 immunoreactivity can be detected in astrocytes, microglia, and neurons in the penumbra [38]. In the central nervous system, investigators have confirmed astrocytes to be major antioxidant resources that protect neurons against oxidative damage [39–41]. Furthermore, in mixed neuron/astrocyte cultures subjected to oxygen-glucose deprivation, astrocytes are the sole locus for Nrf2 activation in response to oxidative stress [42, 43]. Interestingly, a new study has shown that astrocytes with mutant SOD1 increase P-glycoprotein in endothelial cells in vitro [44]. Moreover, astrocytic Nrf2 activation helps to prevent oligodendrocyte loss and demyelination in an animal model of multiple sclerosis [45]. These data suggest that induction of Nrf2 in astrocytes contributes to neuroprotection, BBB repair, and white matter recovery after various brain injuries. In contrast, Nrf2 expression in neurons is much lower than that in astrocytes [46], a fact that supports the important role of the astrocytic Nrf2 pathway in the brain. However, our knowledge about the role of astrocytic Nrf2 under hemorrhagic conditions is limited. Hemoglobin increases TNF-α and

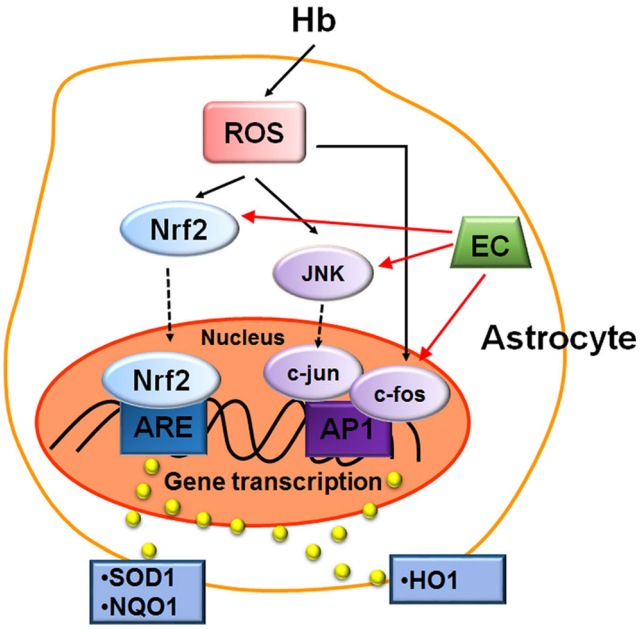

Fig. 5 (−)-Epicatechin protects astrocytes against hemoglobin toxicity via Nrf2 and AP-1 signaling pathways. In astrocytes exposed to hemoglobin (Hb), EC treatment increases expression of cytoplasmic and nuclear Nrf2, which upregulates downstream target proteins SOD1 and NQO1. In addition, EC treatment inhibits AP-1 activity by downregulating JNK phosphorylation and nuclear expression of JNK/c-jun/c-fos, which could be another target of EC. In hemoglobin-exposed astrocytes, EC may fail to alter HO1 expression because the opposing effects of Nrf2 activation and AP-1 inhibition may produce a net zero response. *ARE* antioxidant response element, *HO1* heme oxygenase 1, *NQO1* NAD(P)H oxidoreductase 1, *ROS* reactive oxygen species, *SOD1* superoxide dismutase 1

decreases TGF-β level by dose-dependently activating NF-κB in human astrocytes exposed to hypoxia [47]. To explore the role of astrocytic Nrf2 in ICH, we chose hemoglobin-injured primary astrocytes in vitro to simulate acute post-ICH conditions. In the present study, we assessed the effects of hemoglobin on astrocytes after 2 and 24 h of exposure and found that hemoglobin stimulated Nrf2 nuclear translocation in WT astrocytes and induced ROS production in Nrf2 KO astrocytes at a level almost twice that in WT astrocytes. This finding strongly implies a critical antioxidant role for astrocytic Nrf2 signaling after ICH. To further study Nrf2 activation and signaling in ICH, we will consider using a neuron-astrocyte coculture system to investigate Nrf2-mediated neuroprotection in our future studies.

EC is a natural flavonoid compound that is enriched in green tea and cocoa. Recent studies have shown that EC has the capability of crossing the BBB in rats [25] and of being transported across BBB cells (RBE-4, immortalized cell line of rat capillary cerebral endothelial cells; hCMEC/D3, immortalized human cerebral microvessel endothelial cell line) at a concentration of 30 μM [26]. Moreover, EC enhanced ARE expression in Nrf2 overexpressed astrocytes [48]. However, the effects of EC on astrocytes stimulated with hemoglobin are unknown. Furthermore, our previous in vivo study showed that EC mitigates histologic and neurologic deficits after ICH in both WT and Nrf2 KO mice, suggesting that EC may act through Nrf2-dependent and Nrf2-independent pathways and could be a novel drug candidate for ICH treatment [7]. Here, we showed that EC increased cytoplasmic and nuclear Nrf2 expression and protected WT astrocytes after hemoglobin exposure. Interestingly, EC also protected Nrf2 KO astrocytes against hemoglobin toxicity. In combination, these data suggest that EC exerts protective effects via Nrf2-dependent and Nrf2-independent processes in animal models of ICH and in astrocytes exposed to hemoglobin.

One notable finding from this study is that although SOD1 and HO1 are both downstream of Nrf2, effects of EC on these two proteins are quite different. EC increased SOD1 expression in WT but not in Nrf2 KO astrocytes. This result indicates that upregulation of SOD1 expression by EC is Nrf2-dependent in astrocytes exposed to hemoglobin. In contrast, EC did not upregulate HO1 expression in hemoglobin-stimulated WT astrocytes but decreased HO1 expression in Nrf2 KO astrocytes. This finding indicates that the other targets of EC may regulate HO1 expression. Interestingly, the role of HO1 after ICH is controversial [5]. In ICH patients, HO1 expression in the hemorrhagic brain is associated with CD163 expression at 72 h after ICH [49], and serum HO1 level is increased in ICH patients [50]. In an animal model of ICH, systemic hemin therapy was reported to increase HO1 expression and attenuate blood-brain barrier disruption [51]. Additionally, porphyrin nonselective HO1 inhibitors have consistently been protective in animal models of ICH [52–54], and downregulation of HO1 by valproic acid protects against hemin toxicity [55]. Moreover, we have reported previously that HO1 deletion in mice reduces early ICH injury [56]. Therefore, the effect of EC on HO1 expression after ICH seems to be complicated and not entirely Nrf2-dependent.

AP-1 is a stress-activated transcription factor that has been implicated in ischemic stroke and traumatic brain injury [14]. In fibroblasts from Nrf2 KO mice, HO1 expression is increased by arsenite treatment, probably via AP-1 activation [57]. In astrocytes, high glucose-induced HO1 expression is regulated by AP-1 activation and contributes to neuronal apoptosis [58]. AP-1 is also involved in the regulation of matrix metalloproteinase-9 [59], which contributes to early ICH injury [60, 61]. Here, we showed that in astrocytes from WT and Nrf2 KO mice, EC treatment decreased the nuclear expressions of JNK, c-jun, and c-fos after hemoglobin exposure, which implies that EC strongly inhibits AP-1 transcription. Therefore, the failure of EC to alter HO1 expression in WT astrocytes after hemoglobin exposure may result from a

balance between increased Nrf2 activity and decreased AP-1 activity. In contrast, our previous in vivo study showed that EC decreases HO1 expression level in WT mice after ICH [7]. Considering that our current study examined astrocytes alone in vitro, the effects of hemoglobin with and without EC treatment on other cell types such as neurons, microglia, and oligodendrocytes need to be explored in the future. The interactions between these different cell types will be important for explaining the overall action of EC on the ICH brain.

In conclusion, we identified two potential targets of EC in astrocytes exposed to hemoglobin. Nrf2-ARE/SOD1 is an important signaling pathway by which EC protects astrocytes against hemoglobin toxicity. Furthermore, hemoglobin can activate AP-1 signaling, and EC-induced inhibition of this pathway can provide complementary protection even in Nrf2 KO astrocytes. These cellular and molecular effects provide additional evidence to support EC's cerebroprotective effects as we previously observed in animal models of ICH. As a transcription factor that regulates inflammation, cell death, and cell proliferation, AP-1 could be a new target for ICH treatment.

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Compliance with Ethical Standards The authors declare that they have no conflict of interest.

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