The novel prolyl hydroxylase-2 inhibitor caffeic acid upregulates hypoxia inducible factor and protects against hypoxia

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Abstract

Background & aims: Hypoxia inducible factor (HIF) is a hypoxia-associated transcription factor that has a protective role against hypoxia-induced damage. Prolyl hydroxylase-2 (PHD2) is a dioxygenase enzyme that specifically hydroxylates HIF targeting it for degradation, therefore, inhibition of the PHD2 enzyme activity acts to upregulate HIF function. This study was to identify novel PHD2 inhibitors.

Methods: An established fluorescence-based PHD2 activity assay was used for inhibitors screening. Western blot and quantitative real-time PCR was used to detect the protein and mRNA levels respectively. Further animal experiment was carried out.

Results: Caffeic acid was screened and identified as a novel PHD2 inhibitor. Caffeic acid treated PC12 and SH-SY5Y neuronal cell lines stabilized endogenous HIF-1α protein levels and consequently increased mRNA levels of its downstream regulated genes *VEGF* and *EPO*. Caffeic acid treatment reduced hypoxia-induced cell apoptosis and promoted HIF/BNIP3-mediated mitophagy. Moreover, animal studies indicated that caffeic acid increased the level of HIF-1α protein and mRNA levels of *VEGF* and *EPO* in the brain of mice exposed to hypoxia. Conventional brain injury markers including malondialdehyde, lactic acid and lactate dehydrogenase in the caffeic acid treated mice were shown to be reduced to the levels of the control group.

Conclusions: This study suggests that caffeic acid inhibits PHD2 enzyme activity which then activates the hypoxia-associated transcription factor HIF leading to a neuroprotective effect against hypoxia.

Keywords

PHD2, HIF, caffeic acid, hypoxia, mitophagy

1. Introduction

Hypoxia inducible factor (HIF) is a hypoxia-associated transcription factor that modulates the expression of genes involved in processes such as angiogenesis, erythropoiesis and metabolism (Chowdhury et al., 2008). HIF has a protective effect due to the induced expressions of various anti-apoptotic proteins under hypoxic conditions. HIF-1 consists of an α/β heterodimer, while HIF-1α and HIF-1β are the regulatory and constitutively active subunits respectively. The regulatory HIF-1α subunit is catalyzed by the prolyl hydroxylase-2 (PHD2) enzyme, resulting in hydroxylation on Pro402 and Pro564 residues (Abboud et al., 2018). During normoxia, the proline residues in the oxygen dependent degradation (ODD) domain of HIF-1α is hydroxylated, which promotes its binding to the von Hippel Lindau protein (VHL)-elongin B-elongin C (VBC) protein complex, leading to ubiquitination and rapid degradation in a proteasome-dependent way with a half-life of 5 min (Rytkönen et al., 2011). Under hypoxic conditions, there are insufficient quantities of oxygen and the activity of PHD2 is inhibited resulting in an upregulation of HIF-1α (S. Y. Kim & Yang, 2015). The stabilized HIF-1α in this situation then translocate towards the nucleus to activate more than 70 known target genes containing hypoxia response elements (HREs) in their upstream promoter regions (Benita et al., 2009; Lando et al., 2002; Schofield & Ratcliffe, 2004). The inhibition of PHD2 has been proposed as a promising therapy target for HIF-related diseases, such as ischaemic brain injury and anemia, by regulating HIF protein levels (Davis et al., 2018).

Autophagy is a process defined by the degradation of abnormal proteins and damaged organelles through autolysosomes formed by autophagosome–lysosome fusion to maintain cell homeostasis (Parzych & Klionsky, 2014; Tagaya & Arasaki, 2017). Mitophagy is a type of autophagy that

selectively degrades damaged mitochondria through an autophagy mechanism to sustain the stability of the intracellular environment (Glick et al., 2010). The B Lymphoma-2 gene/adenovirus E1B interacting protein 3 (BNIP3) is a pro-apoptotic mitochondrial protein located in the outer membrane of mitochondria (Kubli et al., 2007; H. Liu et al., 2019), which interacts/recruits LC3 to form autophagosomes to clear the damaged mitochondria (Roperto et al., 2019; Springer & Macleod, 2016; Šprung et al., 2018). HIF-1α up-regulates BNIP3 expression which contributes to the autophagic initiation by disrupting Beclin-1 from the Bcl-2/Beclin-1 complex and protects cells from apoptosis by curbing the mitochondria balance (Tang et al., 2020).

Caffeic acid is a polyphenol and a secondary metabolite obtained from natural sources including coffee beans, olives, berries, potatoes and carrots (Silva et al., 2014; Stojković et al., 2013). It is a constituent of hydroxycinnamic acid, which is a common everyday dietary ingredient for humans (Agunloye & Oboh, 2018). Caffeic acid has been broadly employed as an antioxidant (J. H. Chen & Ho, 1997; Genaro-Mattos et al., 2015). In addition, caffeic acid has been used to treat microbial infections (Ikeda et al., 2011; Kwon et al., 2007) and is well recognized as an antithrombotic, antihypertensive, antidiabetic, anticancer and anti-inflammatory agent (Bhullar et al., 2014; Jung et al., 2006; Matejczyk et al., 2018; Nam et al., 2020; Yang et al., 2013).

We have previously produced highly active recombinant human PHD2 protein in insect cells and established a fluorescence-based PHD2 activity assay for high-throughput screening of PHD2 small molecule inhibitors (Guo et al., 2022). In this study, we have identified caffeic acid as a novel PHD2 inhibitor by screening of a natural compound library. Further *in vitro* and *in vivo* studies indicated

that caffeic acid was shown to effectively inhibit PHD2 enzyme activity, upregulate the level of HIF-1α protein and had a neuroprotective effect against hypoxia-induced damage.

2. Materials and methods

2.1. Screening of PHD2 small molecule inhibitors

A fluorescence-based PHD2 activity assay for high-throughput screening of PHD2 small molecule inhibitors was previously established (Guo et al., 2022). To screen for inhibitors of PHD2, 1 mM natural compound (Natural Product Library, Selleck, USA) stock solutions were diluted to a final concentration of 80 μM in a reaction buffer (Tris-HCl 20 mM, pH 8.0, 20 μM α-ketoglutaric acid and 2 mM Vitamin C) and incubated with 0.5 μM purified recombinant GST-PHD2 for 30min. Subsequently, 1 μM FITC-labeled HIF1α peptide (DLDLEALAPYIPADDDFQLR) was mixed and added into black well plates. After 1h at room temperature, purified von Hippel Lindau protein (VHL)-elongin B-elongin C (VBC) protein complexes were then added into the reaction system and enzymatic activity was measured using a microplate reader (SYNERGY 4, Bio-tek). Excitation at 485 nm and emission at 528 nm were used to obtain the fluorescence polarization values.

2.2. Molecular docking analysis

AutoDock version 4.2 software was performed to analyze the free energy binding estimations using a standard protocol. The 3D structure of PHD2 protein was obtained from the Protein Data Bank (code 6nmp), and caffeic acid was obtained from PubChem. A PDBQT file of PHD2 protein containing a protein structure with hydrogens in all polar residues was created. All compound bonds were set to be rotatable. All calculations were done using the Lamarckian Genetic Algorithm (LGA) method.

For docking studies, the global search exhaustiveness was set up at 100, and the docking poses predictions of each compound towards PHD2 protein was calculated from each energy minima. The average affinity for best poses was taken as the final affinity value.

2.3. Cell culture

All cell lines were obtained from the American Type Culture Collection (ATCC). Rat pheochromocytoma PC12 and Human neuroblastoma SH-SY5Y cell lines were cultured respectively in RPMI 1640 (Gibco, Cat. No. 31800) or DMEM (Gibco, Cat. No. 12100046) each supplemented with 10 % FBS (EVERY GREEN), 100 U/ml penicillin/0.1 mg/ml streptomycin (Beyotime) in a humidified atmosphere with 5 % CO_2 at 37°C.

2.4. Cell viability and apoptosis assay

Cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After drug or vehicle control treatment, cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Solarbio, Beijing, China) dye absorbance and expressed as the percentage of non-treated cells. Briefly, MTT solution (20 μl, 0.5 mg/ml in PBS) was added into each cell dish and incubated at 37°C for 4 h. Then, DMSO (200 μl/well) was added to dissolve the formazan dyes and the absorbance was measured at 490 nm using a microplate reader (Bio-Rad, USA).

Treated cells were collected and washed with PBS (20 mM, pH 7.4), and cell apoptosis was measured using an Annexin V-FITC Apoptosis Detection Kit (BestBio, ShangHai, China) following the manufacturer's instructions. Cells were detected by flow cytometry and data were analyzed using

an Accuri C6 flow cytometer (BD Biosciences, CA, USA).

2.5. Western blot

Cell lysates were extracted and separated by 12 % SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% non-fat milk in Tris-buffered saline containing 0.1 % Tween-20 (TBST), the membranes were incubated with primary antibody at 4°C overnight. After washing with TBST, the membranes were incubated for an additional 2 h with the appropriate secondary antibodies conjugated to HRP. The immunoblot bands were visualized using a chemiluminescence analysis system (SageCreation, Beijing, China). The density of the bands were analyzed using ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA).

Rabbit anti-HIF-1α monoclonal antibody (Cat. No. 36169; 1:1000) was purchased from Cell Signaling Technology (USA). Mouse anti-PHD2 monoclonal antibody (Cat. No. sc-271835; 1:200), mouse anti-BNIP-3 monoclonal antibody (Cat. No. sc-56167; 1:200) and mouse anti-Beclin-1 monoclonal antibody (Cat. No. sc-48341; 1:200) were purchased from Santa Cruz Biotechnology (USA). Rabbit anti-LC3 B polyclonal antibody (Cat. No. L7543; 1:1000) was purchased from Sigma-Aldrich (USA). Rabbit anti-β-Actin monoclonal antibody (Cat. No. AC026; 1:20000) was purchased from ABclonal Technology (China), Rabbit anti-Histone H3 polyclonal antibody (Cat. No. AF0863; 1:5000) was purchased from Affinity Biosciences (China). Mouse anti-GAPDH monoclonal antibody (Cat. No. KM9002; 1:5000), goat anti-Mouse IgG(H+L)-HRP (Cat. No. LK2003; 1:5000) and goat anti-Rabbit IgG(H+L)-HRP (Cat. No. LK2001; 1:5000) were purchased from Sungene Biotech (China).

2.6. Quantitative real-time PCR

Total RNA was extracted from cells using the TRIzol® Reagent (Life Technologies, California, USA), and reverse transcription was performed using the All-in-One First-Strand cDNA Synthesis SuperMix kit for RT-PCR (TransScript. Beijing, China). mRNA levels were detected using quantitative real-time PCR analysis with SybrGreen qPCR Mastermix (DBI Bioscience. Shanghai, China). The reaction was carried out at 95°C for 2 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec, 72°C for 30 sec and 95°C for 1 min. The sequences (written from 5' to 3') of primers used for RT-PCR were as follow: *VEGF* (Human) forward, GTCCCTCTTGGAATTGGATT; *VEGF* (Human) reverse, GTATGTATGTGGGTGGGTGT; *EPO* (Human) forward, CCCCACCACGCCTCATCTGT; *EPO* (Human) reverse, TGCTGCCCGACCTCCATCCT; *β-actin* (Human) forward, GTTGCGTTACACCCTTTCTT; *β-actin* (Human) reverse, CACCTTCACCGTTCCAGTTT; *VEGF* (Mouse) forward, CAGGCTGCACCCACGACAGA; *VEGF* (Mouse) reverse, CACGCACTCCAGGGCTTCAT; *EPO* (Mouse) forward, AAAGAATGGAGGTGGAAGAA; *EPO* (Mouse) reverse, GTACCCGAAGCAGTGAAGTG; *β-actin* (Mouse) forward, CTAAGGCCAACCGTGAAAAG; *β-actin* (Mouse) reverse, ACCAGAGGCATACAGGGACA;

2.7. Animal experiments

The animal experiments in this study received approval (2021-Shengwu-001) from the Ethics Committee of the Tianjin University of Science and Technology, Tianjin, China. C57BL/6J mice (6-week-old) were purchased from the National Institutes for Food and Drug Control (Beijing, China

quality certification number: SCXK (Jing) 2017-0005). All mice were fed *ad libitum* and housed under controlled lighting conditions (12 light: 12 dark). All mice were randomly divided into control, vehicle and experimental groups respectively containing five animals per group. The mice in the experimental group were fed with 500 mg/kg caffeic acid (200 µl in 0.9 % NaCl) daily via oral gavage for a period of 7 days. The vehicle group were fed with 200 µl 0.9 % NaCl daily via oral gavage for a period of 7 days. Then the mice in vehicle group and experimental group were exposed to hypoxia (5 % O_2) for 4 h. At the end of the treatment, all mice in the three groups were sacrificed and the brain of each mouse was carefully dissected out and washed with physiological saline. The organ was snap-frozen in liquid nitrogen for further analysis.

1 ml pre-cooled 0.9 % NaCl buffer was added onto 100mg of brain sample. The tissue was then ground and the lysate centrifuged at 2500 rpm for 10min. The clarified supernatant was then removed and the concentrations of malondialdehyde (MDA), lactic acid (LD) and lactate dehydrogenase (LDH) were measured using a Malondialdehyde (TBA method) (Cat. No.: A003-1-2), Lactic Acid (Cat. No.: A019-2-1) and Lactate dehydrogenase assay kit (Cat. No.: A020-2-2) respectively. All these assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.8. Statistical analysis

Quantitative data were expressed as mean ± SD. Student's unpaired test were used to compare the differences between groups using GraphPad Prism 8.3.0 (GraphPad Software, USA). P values <0.05 were considered statistically significant.

3. Results

3.1. Screening and identification of PHD2 small molecule inhibitors

To screen for small molecule inhibitors of PHD2, we established a PHD2 enzymatic activity assay method for high-throughput screening using functional recombinant human PHD2 protein expressed in insect cells (Guo et al., 2022). By screening a 120 compound library set (Natural Product Screening Library, Selleck, USA), caffeic acid was shown to have the largest inhibitory effect on PHD2 activity (Fig. 1A). In addition, caffeic acid showed a concentration dependent inhibition of PHD2 activity with an IC_{50} of 0.16 μM (Fig. 1B).

To further determine the molecular mechanism of how caffeic acid inhibits PHD2 enzyme activity, the binding mode and interaction of PHD2 protein with caffeic acid was estimated using molecular docking analysis. The binding modes were visualized using PyMOL (version 5.10.130.0) and Ligplot software (version 2.1) in three and two-dimensional representations respectively. Ligplot analysis revealed that residues Met299, Tyr303, Tyr310, His313 and Asp315 were important residues for PHD2 interacting with this compound (Fig. 1C). The major bond type of conjugation in the formation of this complex was conventional hydrogen bond interactions. The binding energy value (ΔG) and inhibitory constant of PHD2-caffeic acid complex were −3.95 kcal/mol and 1.28 mM respectively.

3.2. Caffeic acid stabilizes endogenous HIF-1 protein levels and promotes its activation

We further determined the capability of the identified PHD2 inhibitor caffeic acid in regulating HIF-1α within a cellular context. The cytotoxicity of caffeic acid against the rat pheochromocytoma

cell line PC12 and the human neuroblastoma cell line SH-SY5Y was firstly assessed using an MTT assay. As shown in Fig. 2, treatment with less than 500μM caffeic acid for 24 hours did not show cytotoxicity on both PC12 and SH-SY5Y cells. Interestingly, caffeic acid treatment promoted the cell viability of SH-SY5Y cells (Fig. 2B), which indicates that caffeic acid is a safe natural compound with very low cytotoxicity.

To verify whether caffeic acid stabilizes HIF-1α protein levels in these cells, 250 μM and 500 μM caffeic acid were added to the culture medium respectively and treated for 24 hours. The treated cells were then collected for western blot analysis. In comparison to the control, 500 μM caffeic acid increased the protein levels of endogenous HIF-1α about 3-fold in PC12 cells (Fig. 2C) and approximately 4-fold in SH-SY5Y cells (Fig. 2D). The levels of endogenous PHD2 protein however did not noticeably change in both cell lines (Fig.2 C, D).

Activation of transcription factor HIF-1α protein results in its binding to the hypoxia responsive element (HRE) motif on gene promoters, thereupon promoting the expression of downstream genes such as *VEGF* and *EPO*. To further determine the effect of caffeic acid treatment on the activation of HIF-1α, the mRNA levels of *VEGF* and *EPO* in SH-SY5Y cell line were measured. As shown in Fig. 2E, after 500 μM caffeic acid treatment for 24 hours, the mRNA levels of *VEGF* and *EPO* showed approximately 3-fold and 1.5-fold increase respectively. These results indicated that the PHD2 inhibitor caffeic acid stabilized HIF-1α and also stimulated its activation as a transcription factor.

3.3. Caffeic acid reduces hypoxia-induced cell apoptosis

Hypoxia signaling pathway studies have shown that hypoxia induces cell apoptosis (Saraste & Pulkki, 2000). To determine whether the observed caffeic acid induced activation of HIF-1α alleviates hypoxia-induced cell apoptosis, two neuronal cell lines were pre-treated with caffeic acid for 12 hours, and then changed to glucose free DMEM medium with 0.1 % Na_2SO_3 for 12 hours, which mimicked the hypoxia environment in cell culture (H. Kim & Kwon, 2021). MTT was then used to monitor cell viability. As shown Fig. 3A, compared to the control, there was a significant increase in cell viability of PC12 cells with caffeic acid pre-treatment, reaching to about 2-fold the level observed under 250 μM caffeic acid treatment. The cell viability of SH-SY5Y cells also showed a 2-fold increase after 250 μM caffeic acid pre-treatment compared to the control (Fig. 3B).

Furthermore, flow cytometry was performed to analyze the degree of apoptosis of SH-SY5Y cells cultured in conditions that mimic hypoxia with or without caffeic acid treatment (Fig. 3C). The results showed that the percentage of apoptotic cells was approximate 30 % in the hypoxia mimic condition, while the percentage of cells undergoing apoptosis was reduced to about 8 % after pre-treatment with 250 μM caffeic acid.

3.4. Caffeic acid promotes the BNIP3/Beclin-1 signaling pathway induced-mitophagy

It has been reported that PHD2 inhibitors induce autophagy and protect PC12 cells grown in oxygen-glucose deprivation conditions (Singh et al., 2020). We thereafter decided to monitor the effect of caffeic acid on mitophagy. PC12 and SH-SY5Y cell lines were treated with caffeic acid for 24 hours, the cells were then collected and analyzed by western blot. Subsequent western blot analysis showed that caffeic acid treatment resulted in a significant increase of BNIP3 and Beclin-1

protein levels. The ratio of LC3-II/LC3-I (conversion of soluble LC3-I to lipid bound LC3-II) was also significantly increased in a dose-dependent manner (Fig. 4A), which indicated that caffeic acid treatment promoted mitophagy, a type of autophagy. Consistently, similar results were also obtained for the caffeic acid treatment of SH-SY5Y cells (Fig. 4B).

3.5. Caffeic acid reduces hypoxia-induced brain injury in mice

In order to verify whether caffeic acid promotes activation of HIF-1α in vivo, male C57BL/6J mice (6-week-old) were fed with 500 mg/kg caffeic acid daily via oral gavage for a period of 7 days, and the mice in the control group were fed normally. RT-PCR analysis showed that the mRNA levels of *VEGF* and *EPO* in the mice brains were significantly increased after caffeic acid treatment compared to the control group (Fig. 5A). The endogenous HIF-1α protein levels in the brain in both group mice were very low and showed no clear difference, probably due to quick degradation of HIF-1α under normoxic conditions.

Our studies have shown that caffeic acid promotes mitophagy and reduces hypoxia-induced cell apoptosis of cultured neuronal cell lines. We then investigated whether caffeic acid has a protective effect on brain injury in mice under hypoxic environmental conditions. Male C57BL/6J mice (6-week-old) were randomly divided into three groups. The mice in the experimental group were fed with 500 mg/kg caffeic acid daily via oral gavage for a period of 7 days. The vehicle group were fed with 0.9 % NaCl. Then the mice were exposed to hypoxia (5% O_2) for 4 hours (Fig. 5B). As shown in Fig. 5C, the endogenous HIF-1α protein levels in brain in the control group mice were relatively low, while the endogenous HIF-1α protein in the mice exposed to hypoxia was significantly

increased. Interestingly, mice treated with caffeic acid showed even higher HIF-1α protein levels (about 2-fold) than mice in the vehicle group. Consistent with the data from caffeic acid treated cultured neuronal cells, these results indicated that caffeic acid treatment of mice had increased levels of HIF-1α protein in their brains.

Moreover, RT-PCR was performed to analyze the mRNA levels of HIF-1α targeting genes in the mice brains. As shown in Fig. 5D, the mRNA levels of *VEGF* and *EPO* in the mice brains were significantly increased after caffeic acid treatment. These results indicated that the PHD2 inhibitor, caffeic acid, activated HIF-1α in mice brains. Furthermore, conventional brain injury markers including MDA, LD and LDH were analyzed to evaluate the protective effect of caffeic acid treatment on hypoxia-induced brain injury. Under hypoxic conditions, MDA, LD and LDH levels in mice without caffeic acid treatment were markedly up-regulated. However, the levels of these brain injury markers in the caffeic acid treated mice were significantly decreased compared to the levels of the control group (Fig. 5E).

4. Discussion

Hypoxia is generally associated with human disease processes related to anemia, ischemia, most tumors, and other hypoxic-ischemic diseases, making HIF-1α an attractive target for therapeutic intervention (Higashijima et al., 2013; Jain et al., 2018). As PHD2 is a regulatory enzyme of HIF-1α, PHD2 inhibitor compounds have been studied in stroke models either *in vivo* or *in vitro*, where they have shown neuroprotective effects following an ischaemic insult (R. L. Chen et al., 2014; Nagel et al., 2011; Ogle et al., 2012; Reischl et al., 2014; Zhao & Rempe, 2011; Zhou et al., 2017). In this

study, caffeic acid was identified as a novel inhibitor of PHD2. In addition, ferulic acid and isoferulic acid, as metabolites of caffeic acid *in vivo* (Omar et al., 2012), did not exhibit inhibition on PHD2 activity (data not shown). Moreover, molecular docking analysis revealed that caffeic acid interacted with residues Met299, Tyr303, Tyr310, His313 and Asp315, which is the catalytic region of PHD2 (Chowdhury et al., 2020), and is consistent with the site of previously reported inhibitors (Debenham et al., 2016; Li et al., 2019; Zhang et al., 2020). Fe^{2+} is a critical cofactor for the activity of PHD2 and binds to the residues His313, Asp315 and His374 of PHD2 (Zhang et al., 2020), which suggests caffeic acid may compete the iron binding sites for PHD2 inhibition. In addition, caffeic acid phenethyl ester (CAPE) has been shown to activate HIF-1 by inhibiting HIF prolyl hydroxylase (HPH) (Choi et al., 2010; Kim et al., 2015). This study revealed that the catechol moiety of CAPE was essential for HPH inhibition while the double bond and phenethyl moiety assisted in inhibiting HPH, our results indicated that caffeic acid showed direct inhibition of PHD2.

It has been reported that hypoxic preconditioning contributes to the neuroprotection against brain injury in rats via autophagy (Wang et al., 2013; Zhan et al., 2017). Studies have shown that enhancing autophagy had a protective effect against brain damage (Dai et al., 2017; Shen et al., 2016). Mitochondrial dysfunction is a key point leading to brain injury during stroke, and mitophagy, a type of autophagy maintains normal physiological processes by selectively removing dysfunctional mitochondria (Zhong et al., 2022). BNIP3 was closely related to mitophagy, which lead to the accumulation of LC3-II in the reduction of cell damage under hypoxia (Hanna et al., 2012) and also the dissociation of Beclin-1 from Beclin-1/Bcl-2 or Beclin-1/Bcl-XL complexes resulting in the downregulation of apoptosis (Ney, 2015). Our results showed that caffeic acid promoted

HIF-1/BNIP3-mediated mitophagy and protected neuronal cells against hypoxia-induced cell apoptosis.

Caffeic acid is an organic compound that is classified as a hydroxycinnamic acid. It is found in all plants because it is an intermediate in the biosynthesis of lignin, it occurs at high levels in food such as coffee, red wine, argan oil, sunflower seeds and black chokeberry. Though caffeic acid is a type of polyphenol well known for its antioxidant property, recent reports have shown other properties including anti-inflammatory, anti-cancer and antiviral activities. Caffeic acid is a polyphenol found in daily foods, the median lethal dose (LD50) via gavage is 4850 mg/kg in mice (Y. Liu et al., 2019), which indicates the toxicity of caffeic acid is extremely low. Animal experiments showed neuroprotective activity of caffeic acid and plausible permeation through BBB into brain in mice, as the concentration of caffeic acid in human cerebrospinal fluid was almost the same as that in plasma, suggesting caffeic acid can cross the blood-brain barrier in humans and directly affect the functions of brain (Kobylecka et al., 2020). In our study, mice were treated by gavage with 500 mg/kg caffeic. No significant differences were found between the weight and organs of treated mice compared to the control. Analysis of mice brain proteins by Western blot indicated that HIF-1α maintained low steady-state levels due to its short half-life in normoxia, while HIF-1α protein level was significantly increased in the caffeic acid treated mice exposed to hypoxia. The mRNA levels of HIF-1α targeting genes of *VEGF* and *EPO* in the mice brains were significantly increased after caffeic acid treatment under both normoxic and hypoxic conditions. These results suggested that caffeic acid specifically inhibited PHD2 enzyme activity and protected against hypoxia through stabilization and activation of HIF-1.

5. Conclusion

We have identified and demonstrated that caffeic acid inhibits the enzymatic activity of PHD2. This subsequently stabilizes HIF protein levels and activates the hypoxia-associated transcription factor. A reduction in hypoxia-induced cell apoptosis and improvement in hypoxia-induced brain injury in mice are both observed. This study expands the understanding of the bioactivity of caffeic acid in regulating hypoxia protection, as well as the beneficial effects of caffeic acid on health.

Abbreviations

PHD2: prolyl hydroxylase-2; HIF: hypoxia inducible factor; DMEM: Dulbecco's modified Eagle's medium; MDA: Malondialdehyde; LD: Lactic Acid; LDH: Lactate dehydrogenase; ODD: oxygen dependent degradation; VBC: von Hippel Lindau protein (VHL)-elongin B-elongin C; BNIP3: B Lymphoma-2 gene/adenovirus E1B interacting protein 3; LC3: Microtubule-associated proteins 1A/1B light chain 3; VEGF: Vascular endothelial growth factor; EPO: Erythropoietin.

Declaration of interest

The authors declare no conflict of interest.

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Ethics statement animal experimentation

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Tianjin University of Science and Technology and approved by the Animal Ethics Committee of the Tianjin University of Science and Technology, Tianjin, China.

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Figure legends

Fig. 1. Screening and identification of caffeic acid as a novel PHD2 inhibitor. (A) Screening of compounds capable of inhibiting the PHD2 activity from a natural compound library. Caffeic acid showed the highest inhibitory effect, the PHD2 inhibitor IOX2 was used as the positive control. (B) Caffeic acid concentration dependent inhibition of PHD2 activity. The quantification was presented as mean ± SD of three independent experiments. (C) The molecular docking analysis of caffeic acid with PHD2 protein using AutoDock software. The active sites of PHD2 were shown as Met299, Tyr303, Tyr310, His313 and Asp315. Interaction plot by LIGPLOT.

Fig. 2. Caffeic acid stabilizes and activates HIF-1α. PC12 cells (A) and SH-SY5Y cells (B) were treated with caffeic acid at the indicated concentrations for 24 h. Cell viability was determined by MTT assay. HIF-1α and PHD2 protein levels in the caffeic acid treated PC12 cells (C) and SH-SY5Y cells (D) were analyzed by Western blot using antibodies against HIF-1α and PHD2, and densitometric analysis of Western blots was performed using ImageJ software. (E) mRNA levels of *VEGF* and *EPO* in SH-SY5Y cells treated with or without 500 μM caffeic acid were analyzed by RT-PCR. The quantification was presented as mean ± SD of three independent experiments (*P < 0.05, **P < 0.01, ***P <0.001).

Fig. 3. Pre-treatment with caffeic acid reduces hypoxia-induced cell apoptosis. Pre-treatment of PC12 cells (A) and SH-SY5Y cells (B) with caffeic acid at the indicated concentrations for 12 h. Cell viability was determined by MTT assay. The normalized value of cell viability from the cells without hypoxic treatment was arbitrarily set as 100 %. (C) SH-SY5Y cells were treated or without 250 μM

caffeic acid for 12 h, followed by hypoxia mimicking conditions for 12h. The percentage of cell apoptosis was determined using flow cytometry. The control cells were cultured under normal conditions. The quantification was presented as mean ± SD of three independent experiments (*P < 0.05, **P < 0.01).

Fig. 4. Caffeic acid promotes mitophagy. PC12 cells (A) and SH-SY5Y cells (B) were treated with caffeic acid at the indicated concentrations for 24 h. BNIP3, Beclin-1, LC3-I and LC3-II protein levels were monitored by Western blot analysis using antibodies against BNIP3, Beclin-1 and LC3. Densitometric analysis was performed using ImageJ software and the quantification was presented as mean ± SD of three independent experiments (*P < 0.05, **P < 0.01).

Fig. 5. Caffeic acid reduces hypoxia-induced brain injury in mice. (A) mRNA levels of *VEGF* and *EPO* in the brain of mice fed with or without 500 mg/kg caffeic acid daily via oral gavage for a period of 7 days were analyzed by RT-PCR. (B) Experimental timeline schematic for animal experiments including the control (a), vehicle (b) and experimental (c) groups (five mice each group). (C) Western blot analysis was used to detect the protein levels of HIF-1α in the brain of mice (Presented 2 mice each group) and densitometric analysis was performed by ImageJ software. (D) mRNA levels of *VEGF* and *EPO* in the brain of the mice were analyzed by RT-PCR. (E) Concentration determinations of Malondialdehyde (MDA), Lactic Acid (LD) and Lactate dehydrogenase (LDH) in the brain of mice. The quantification was presented as mean ± SD of five mice data in each group (*P < 0.05, **P < 0.01, ***P < 0.001).

Figures

Fig. 1

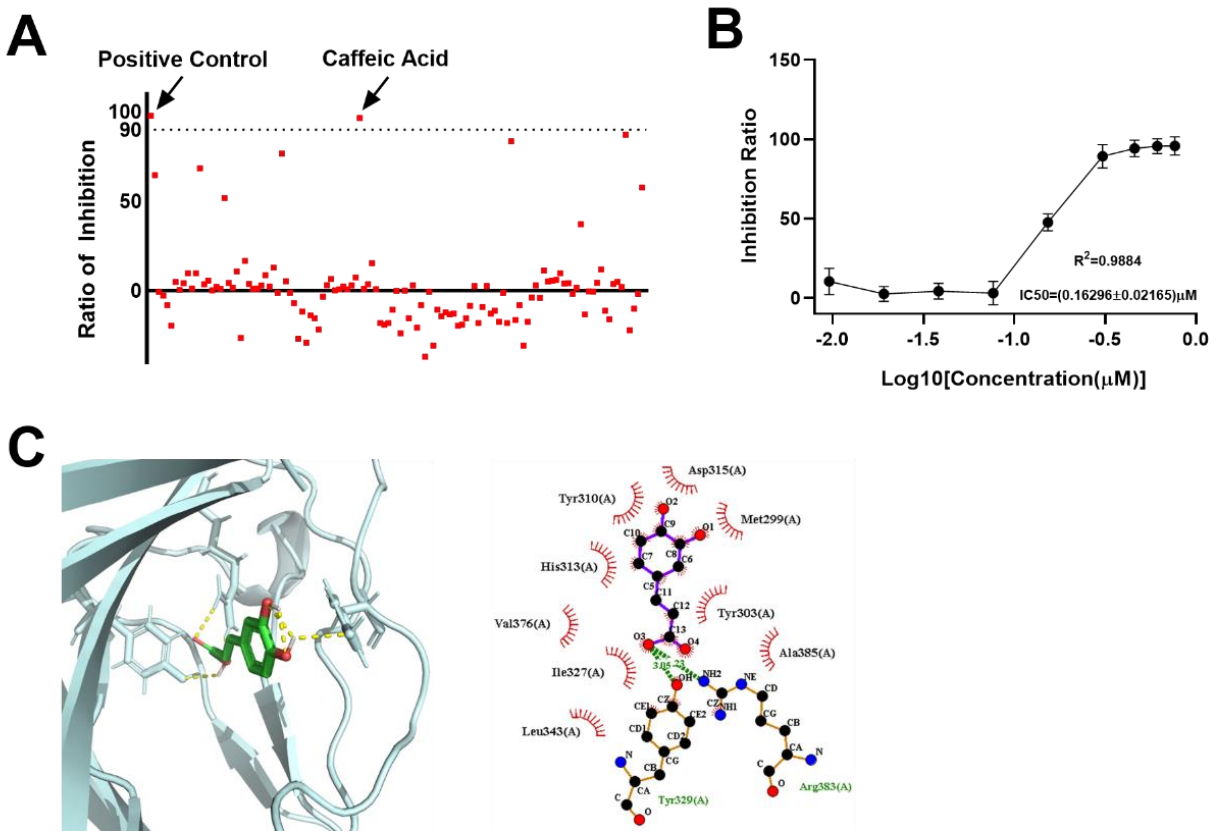

A
Positive Control
Caffeic Acid
Ratio of Inhibition
100
90
50
0
B
Inhibition Ratio
150
100
50
0
R²=0.9884
IC50=(0.16296±0.02165)μM
-2.0
-1.5
-1.0
-0.5
0.0
Log10[Concentration(μM)]
C
Asp315(A)
Tyr310(A)
Met299(A)
His313(A)
Tyr303(A)
Val376(A)
Ala385(A)
Ile327(A)
Leu343(A)
Tyr329(A)
Arg383(A)

Fig. 2

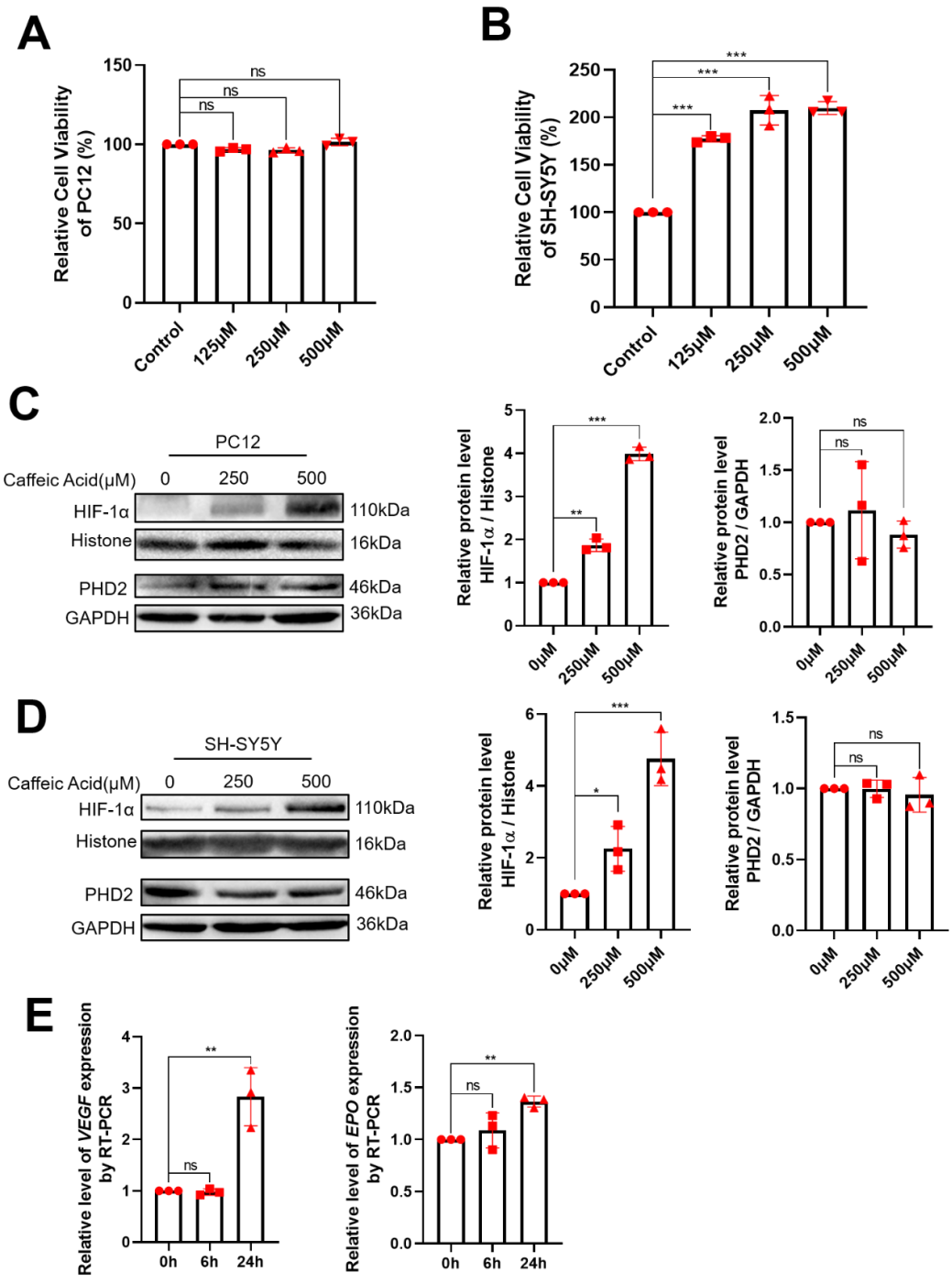

A
Relative Cell Viability of PC12 (%)
150
100
50
0
ns
ns
ns
Control
125μM
250μM
500μM
B
Relative Cell Viability of SH-SY5Y (%)
250
200
150
100
50
0
***
***
***
Control
125μM
250μM
500μM
C
PC12
Caffeic Acid(μM) 0 250 500
HIF-1α
Histone
PHD2
GAPDH
110kDa
16kDa
46kDa
36kDa
Relative protein level HIF-1α / Histone
***
**
0μM
250μM
500μM
Relative protein level PHD2 / GAPDH
ns
ns
0μM
250μM
500μM
D
SH-SY5Y
Caffeic Acid(μM) 0 250 500
HIF-1α
Histone
PHD2
GAPDH
110kDa
16kDa
46kDa
36kDa
Relative protein level HIF-1α / Histone
***
*
0μM
250μM
500μM
Relative protein level PHD2 / GAPDH
ns
ns
0μM
250μM
500μM
E
Relative level of VEGF expression by RT-PCR
**
ns
0h
6h
24h
Relative level of EPO expression by RT-PCR
**
ns
0h
6h
24h

Fig. 3

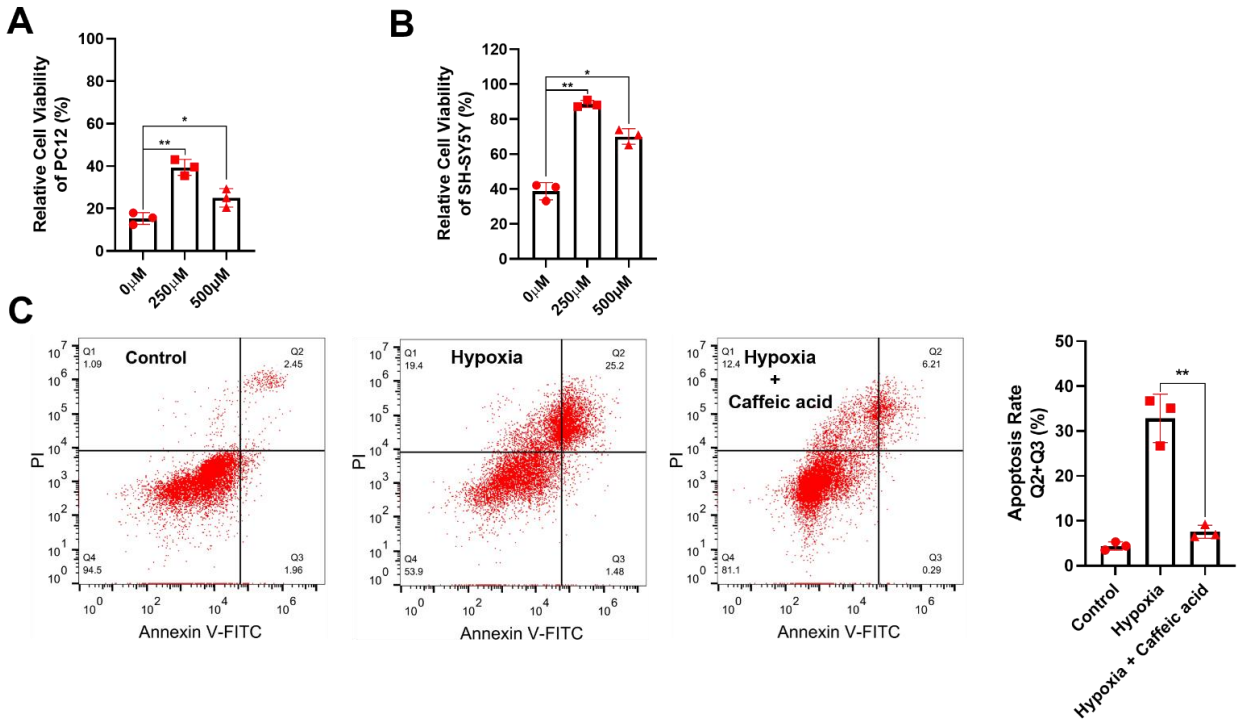

A
Relative Cell Viability of PC12 (%)
0μM
250μM
500μM
B
Relative Cell Viability of SH-SY5Y (%)
0μM
250μM
500μM
C
Control
Hypoxia
Hypoxia + Caffeic acid
PI
Annexin V-FITC
Q1 1.09
Q2 2.45
Q4 94.5
Q3 1.96
Q1 19.4
Q2 25.2
Q4 53.9
Q3 1.48
Q1 12.4
Q2 6.21
Q4 81.1
Q3 0.29
Apoptosis Rate Q2+Q3 (%)
Control
Hypoxia
Hypoxia + Caffeic acid

Fig. 4

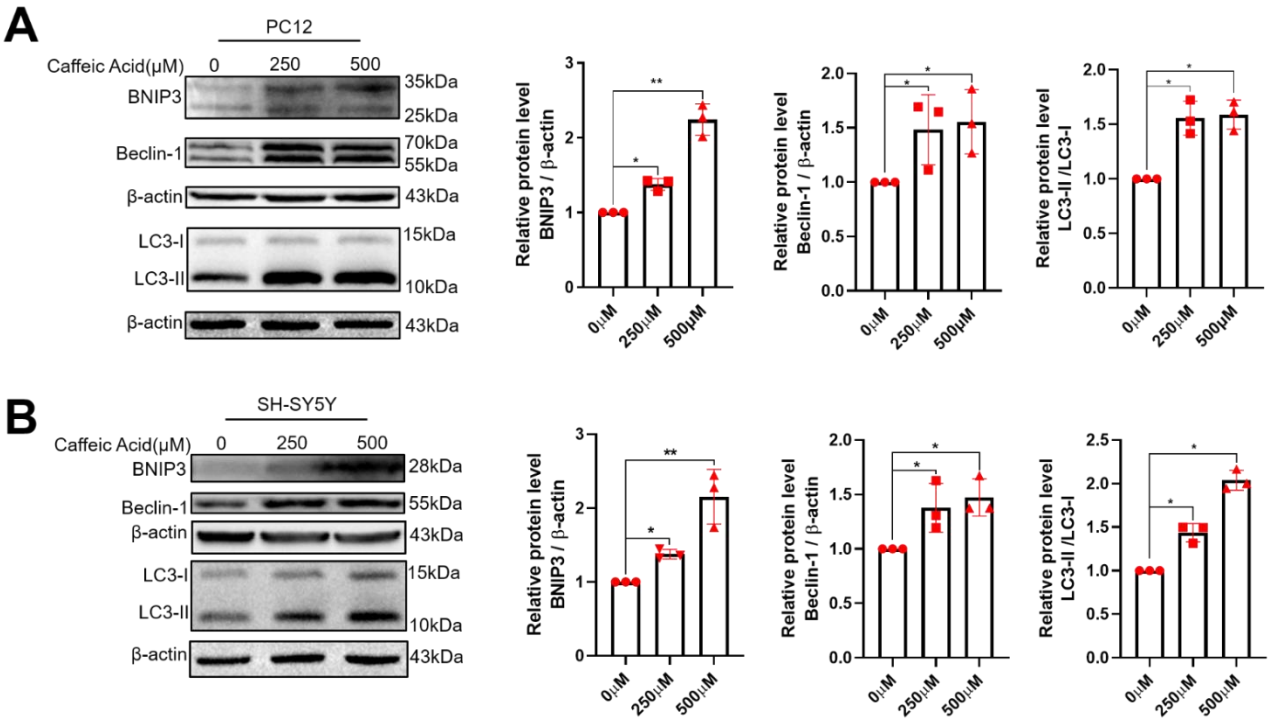
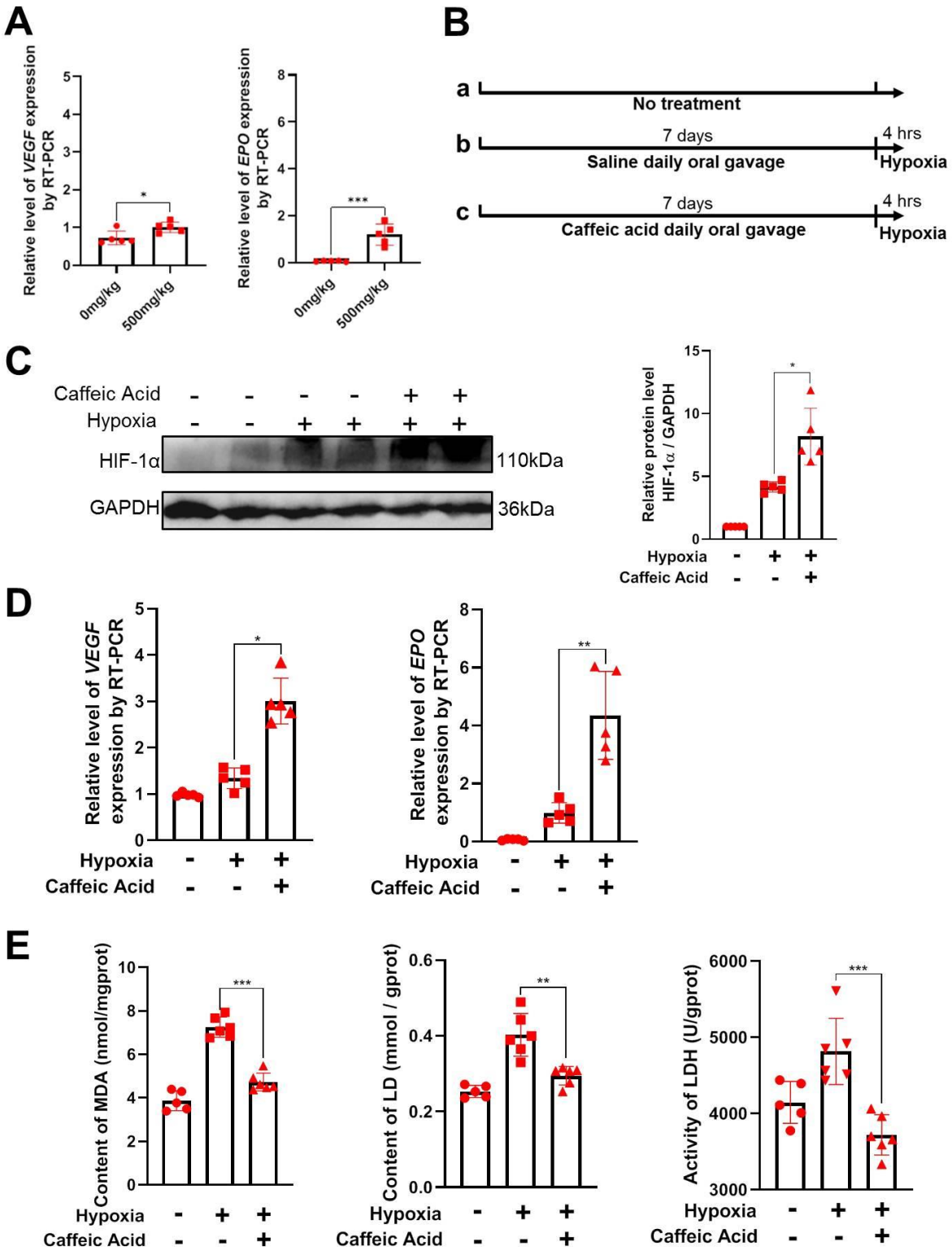
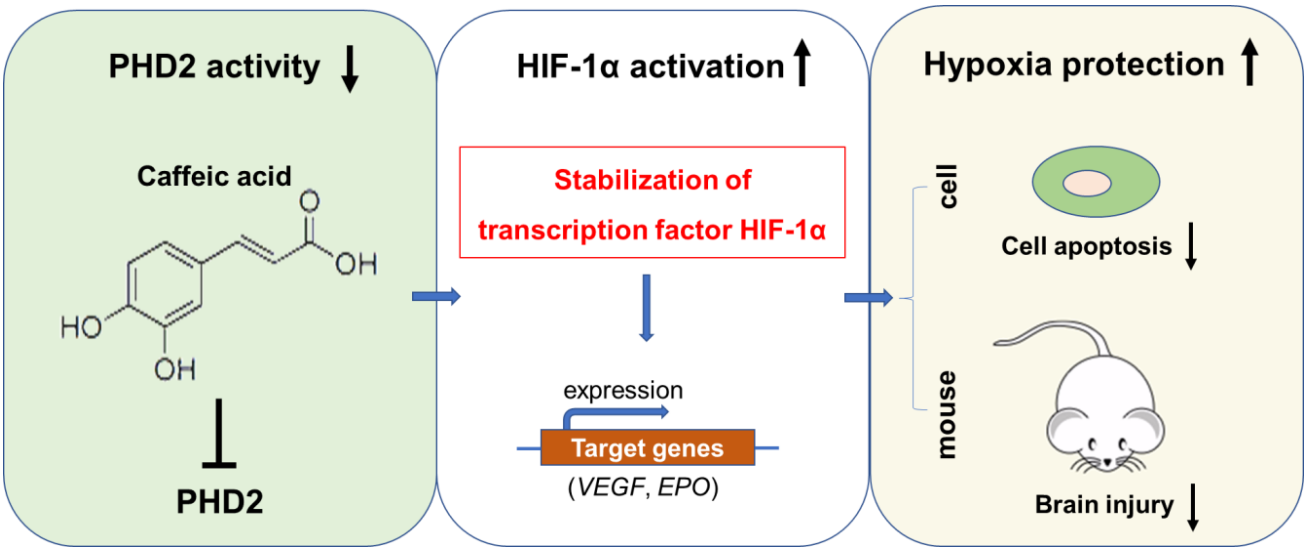

A
PC12
Caffeic Acid(μM) 0 250 500
BNIP3
35kDa
25kDa
Beclin-1
70kDa
55kDa
β-actin
43kDa
LC3-I
15kDa
LC3-II
10kDa
β-actin
43kDa
Relative protein level BNIP3 / β-actin
Relative protein level Beclin-1 / β-actin
Relative protein level LC3-II /LC3-I
0μM
250μM
500μM
B
SH-SY5Y
Caffeic Acid(μM) 0 250 500
BNIP3
28kDa
Beclin-1
55kDa
β-actin
43kDa
LC3-I
15kDa
LC3-II
10kDa
β-actin
43kDa
Relative protein level BNIP3 / β-actin
Relative protein level Beclin-1 / β-actin
Relative protein level LC3-II /LC3-I
0μM
250μM
500μM

Fig. 5



Graphical abstract



Caffeic acid inhibits PHD2 enzyme activity which consequently stabilizes and activates the hypoxia-associated transcription factor HIF-1α. This leads to the protection of hypoxia-induced cell apoptosis and brain injury in mice.