Neuroprotection of hydroxysafflor yellow A in experimental cerebral ischemia/reperfusion injury via metabolic inhibition of phenylalanine and mitochondrial biogenesis

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Abstract. Stroke is the second most frequent cause of mortality, resulting in a huge societal burden worldwide. Timely reperfusion is the most effective therapy; however, it is difficult to prevent ischemia/reperfusion (I/R) injury. In traditional Chinese medicine, hydroxysafflor yellow A (HSYA) has been widely used for the treatment of cerebrovascular disease and as a protective therapy against I/R injury. Evidence has demonstrated that HSYA could reduce the levels of reactive oxygen species and suppress cellular apoptosis; however, whether HSYA alters the metabolic profile as its underlying mechanism for neuroprotection remains unknown. In the present study, using a metabolomic screening, phenylalanine was identified to significantly increase in an experimental model of mouse cerebral I/R injury. Notably, western blotting and qPCR analysis were conducted to test the expression level of apoptosis-associated factors, and HSYA was identified to be able to protect neuronal cells by reducing phenylalanine level associated with I/R injury. Additionally, these findings were confirmed in primary mouse neurons and PC12 cells exposed to oxygen and glucose deprivation/reoxygenation (OGD/R) stress. Of note, HSYA was observed to regulate the mRNA expression of key metabolic enzymes, phenylalanine hydroxylase, tyrosine aminotransferase and aspartate aminotransferase, which are responsible for phenylalanine metabolism. Furthermore, by performing mitochondrial labeling and JC-1 fluorescence assay, HSYA was identified to promote mitochondrial function and biogenesis suppressed by OGD/R. The findings of the present study demonstrated that I/R injury could increase the levels of phenylalanine, and HSYA may inhibit phenylalanine synthesis to enhance mitochondrial function and biogenesis for neuroprotection. The present study proposed a novel metabolite biomarker for cerebral I/R injury and the evaluated the efficacy of HSYA as a potential therapeutic treatment I/R injury.

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Introduction

Cerebral stroke remains the most common cause of mortality and a major cause of neurological disability, resulting in notable societal burden worldwide (1). Ischemia restricts the blood supply to certain regions of the brain, resulting in acute glucose and oxygen deprivation (2). Accumulating evidence demonstrated that ischemia/reperfusion (I/R) stress causes oxidative damage and cell apoptosis (3,4). Increasing efforts have been made to determine the mechanism underlying I/R-associated cell stress and injury (5,6); however, whether I/R injury dysregulates the metabolic profile remains unknown.

In traditional Chinese medicine, safflower (*Carthamus tinctorius*) has been widely used for the treatment of cerebrovascular and cardiovascular diseases (7). The pigments of the *Carthamus tinctorius* extract contain hydroxysafflor yellow A (HSYA), safflor yellow B, safflomin A, safflomin C and other components (8). As the most important and representative component of safflower, HSYA has a notable therapeutic and protective effects against cerebral and myocardial I/R injury (9). HSYA can inhibit cell apoptosis and reduce the levels of reactive oxygen species (ROS), which helps to rescue

damaged neurons and promote cell survival (10). Thus, HSYA is widely used for the neuroprotection for cerebral stroke and I/R in clinic (11).

The 'Warburg effect' has been widely accepted as a common feature of cancer cells that occurs via metabolic reprogramming (12). Of note, metabolic reprogramming is not only limited to cancer cells but has been reported in disorders of the brain, gut and lungs associated with I/R stress (13-15). In addition, cerebral infarction also caused metabolite alterations and reduced the levels of branched-chain amino acid in ischemic stroke (16). It has been demonstrated that HSYA can activate cell survival signaling via the PI3K/AKT pathway, as well as other pathways (17); however, whether HSYA protects against I/R injury via metabolic alterations requires further investigation.

In the present study, we first identified the metabolic amino acid profile in I/R mouse model. I/R injury notably disturbed the metabolic flux and induced a significant increase in levels of plasma phenylalanine. Notably, treatment with HSYA could recover the alterations in phenylalanine levels induced by I/R stress, and regulate the expression of key enzymes, including phenylalanine hydroxylase (PAH), tyrosine aminotransferase (TAT) and aspartate aminotransferase (GOT1), which are responsible for phenylalanine transformation (18). Notably, HSYA was observed to rescue mitochondrial function and glucose uptake ability. Additionally, HSYA could promote mitochondria biogenesis by increasing the expression of fission-associated dynamin-1-like protein (DRP1), which was downregulated via oxygen and glucose deprivation/reoxygenation (OGD/R) and phenylalanine treatment.

The findings of the present study demonstrated the novel mechanism of I/R injury via upregulating the levels of phenylalanine; HSYA was reported to inhibit increases in phenylalanine levels for neuroprotection via enhancing mitochondrial function and biogenesis. The present study proposed a novel metabolite as a biomarker for cerebral I/R injury and the evaluated the efficacy of HSYA treatment.

Materials and methods

Mouse model of ischemia and reperfusion. Male C57BL/6 mice (age, 6-8 weeks; weight, 18-20 g) were obtained from the animal center of The Fourth Military Medical University, and housed with a 12-h light/dark cycle at 18-23˚C, with a humidity of 40-60% and *ad libitum* access to food and water. A total of 24 mice were used to generate a model of cerebral I/R. The mice were divided into four groups: Sham operation and I/R, and I/R model treatment groups, which were treated with HSYA (5 or 20 mg/kg). Transient focal cerebral ischemia was induced by right middle cerebral artery occlusion (MCAO) as described (19). After 2 h, the monofilament was withdrawn to establish the I/R model. Then, two I/R groups of mice were treated with 5 or 20 mg/kg/day HSYA (intraperitoneal injection) for 3 days. Following treatment and analysis of neurological behavior, 50 μl plasma samples were extracted from mouse tail veins, and the mice were subsequently sacrificed. All experiments were approved by The Animal Care Committees of The Fourth Military Medical University (Xi'an, China).

Neurological behavior deficit evaluation. Modified scoring systems for neurological deficits were used to evaluate the neuroprotection effect of HSYA for 72 h with I/R injury. With slight modifications, a 0-4 point scale was used to evaluate the neurological deficits observed in all groups (20). The scoring system used was the following: 0, no deficit; 1, forelimb flexion; 2, forelimb flexion and decreased resistance to lateral push; 3, unidirectional circling; 4, longitudinal spinning, seizure or no movement.

Measurement of infarct zone in the brain. The brain tissues were collected, resected and cut into 2 mm thick slices using a brain matrix, and treated with 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C for 30 min and then fixed in 10% phosphate-buffered formalin for 45 min at room temperature. The tissues were imaged with a light microscope (Olympus CX31; Olympus Corporation, Tokyo, Japan; magnification, 4x). The infarction zone was calculated as the percentage of infarction area compared with the ipsilateral hemisphere. The infarct zone was analyzed in five fields of view using MPLAS-500 multimedia color pathological graphic analysis system (Wuhan Qingping Imaging Technology Co., Ltd., Wuhan, China) (21).

Metabolic profiling and quantification. Samples were analyzed using liquid chromatography-tandem mass spectroscopy (MS/MS). A liquid chromatography system (Shimadzu Corporation, Kyoto, Japan) was used to flow the sample into the MS/MS system. A mixture of acetonitrile and water (70:30, v/v) was used as the carrier solution at a flow rate of 0.14 ml/min. In total, 18 μl of the sample was added to the carrier solution; subsequently, the liquid was delivered to the ion source of the MS/MS system without column separation. The MS/MS analysis was conducted with an API 4000+ tandem mass spectrometer (AB Sciex LLC, Framingham, MA, USA) in positive ionization mode and electrospray ionization was performed with nitrogen gas at 400˚C and the nebulizer pressure was 30 psi. In total, two alternating scan modes were defined in the MS setup. Neutral loss scan of m/z 102 was used for neutral amino acids (25V, 13-17 eV). All data acquisition and processing were performed with Analyst software (version 1.5.2; AB Sciex LLC).

Primary culture of mouse cortex neurons. Pregnant female C57BL/6 mice (age, 8-10 weeks; weight, 18-20 g) were obtained from the animal center of The Fourth Military Medical University (Xi'an, China), housed with a 12-h light/dark cycle at 18-23˚C, with a humidity of 40-60% and *ad libitum* access to food and water. The mice were sacrificed via 50-70% CO_2 inhalation with a fill rate of CO_2 displacement ~20% of the chamber volume/min. Cerebral cortical neurons from the cerebrum of mice at embryonic age 18.5 (E18.5) were prepared. The cerebral cortex of the brains was dissected out in cold PBS and the tissues were cut into small pieces (1 mm^3) and digested with 0.25% trypsin + 0.04% EDTA at 37˚C for 15 min. The cell suspension was centrifuged at 500 x g on 4˚C for 5 min, and the cell pellet was resuspended in Neurobasal + B27 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Following harvest, cells were incubated for 7 days and were subsequently exposed to OGD for 120 min and treated

with or without HSYA at a concentration of 1 or 10 μM for 20 h at 37˚C.

OGD/R in primary mouse neurons. OGD was induced as previously described (22). The cells were cultured with glucose-free Dulbecco's modification of Eagle medium (DMEM; Thermo Fisher Scientific, Inc.) under 5% CO_2 and 95% N_2 at 37˚C for 120 min. Then, the culture medium was replaced with DMEM, and the sample was incubated in regular conditions, with 5% CO_2 at 37˚C for 20 h. In all experiments, the pH of the medium was maintained at 7.2 under OGD conditions.

OGD/R in PC12 cells. Similar to the primary mouse neuronal cells, PC12 cells (American Type Culture Collection, Manassas, VA, USA) were cultured with glucose-free DMEM and infused with 5% CO_2 and 95% N_2 at 37˚C for 12 h. Then, the culture medium was replaced with DMEM, and the sample was returned to the regular conditions of 5% CO_2 and 95% air at 37˚C for 24 h. In all experiments, the pH of the medium was maintained at 7.2 under OGD conditions.

Treatment with phenylalanine in PC12 cells. PC12 cells were treated with various concentrations of phenylalanine (0, 0.5, 1, 2, 4, 8, 16 and 32 mM; Sigma-Aldrich; Merck KGaA) for 48 or 96 h at 37˚C prior to MTT assay. The cells were treated with 8 mM phenylalanine for 48 h in JC-1 prior to western blotting analysis.

Western blot analysis. Radioimmunoprecipitation assay protein extraction buffer was purchased from The Beyotime Institute of Biotechnology (Haimen, China). The total protein was extracted from mouse brain tissues or cultured cells and was subsequently quantified using a bicinchoninic acid assay kit or Bradford's assay kit (Thermo Fisher Scientific, Inc.), respectively. A total of 40 μg protein was loaded per lane. Proteins were separated by 12% SDS-PAGE for 60-80 min. The gel was subsequently transferred on nitrocellulose membranes for 90 min. The membrane was blocked with 5% fat-free milk for 1 h at room temperature. Antibodies against phosphorylated protein kinase B (p-Akt; 1:1,000; cat. no. 4058; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Akt (1:1,000; cat. no. 9272; Cell Signaling Technology, Inc.), anti-cleaved caspase-3 (of c-Casp3; 1:1,000; cat. no. 9664; Cell Signaling Technology, Inc.), anti-β-catenin (1:1,000; cat. no. 8480; Cell Signaling Technology, Inc.), anti-B-cell lymphoma 2 (BCL2; 1:1,000; cat. no. 2870; Cell Signaling Technology, anti-mitochondrial dynamin like GTPase (OPA1; 1:1,000; cat. no. 67589; Cell Signaling Technology, Inc.), anti-voltage-dependent anion channel (VDAC; 1:1,000; cat. no. 4661; Cell Signaling Technology, Inc.), anti-mitofusin 2 (MFN2; 1:1,000; cat. no. 11925; Cell Signaling Technology, Inc.), anti-fission, mitochondrial 1 (Fis1; 1:1,000, ab71498; Abcam, Cambridge, UK) and anti-neuronal nuclei (NeuN; 1:500; cat. no. 26975-1-AP; ProteinTech Group, Inc., Chicago, IL, USA) were applied overnight at 4˚C. β-actin (1:2,000; cat. no. AB8227; Abcam) served as the loading control. Secondary antibodies were incubated for 1 h at room temperature (1:2,000; cat. nos. 7074 and 7076; Cell Signaling Technology, Inc.). Membranes were incubated with chemiluminescent reagents (Pierce; Thermo Fisher Scientific, Inc.) for detecting horseradish peroxidase-labeled antibodies at room temperature prior to x-ray exposure. Western blot analysis was repeated three times, and similar results were obtained. Densitometry was performed using the Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted using TRIzol® RNA Isolation Reagents (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was generated with SuperScript™ II reverse transcriptase according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 2-5 μg RNA was mixed with reverse transcriptase and incubated at 42˚C for 50 min to synthesize cDNA. The mRNA levels of mouse brain tissues, primary mouse neuronal cells or PC12 cells exposed to OGD/R were evaluated using a SYBR-Green RT-qPCR kit (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was conducted using a CFX96 Touch™ RT PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as follows: Denaturation at 95˚C for 10 sec, primer annealing at 60˚C for 20 sec and elongation at 72˚C for 30 sec, for 40 cycles. The mouse and rat-specific primers designed are presented in Table I. The relative quantification of each target gene was normalized to GAPDH using the $2^{-\Delta\Delta Cq}$ quantification method (23), and the fold change between sham and control group was calculated with three replicates in each group.

Flow cytometry and apoptosis analysis. Cell apoptosis was analyzed using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (eBioscience; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. PC12 or primary mouse neuronal cells at 80% confluency were harvested using 0.25% trypsin for 5 min at 37˚C and washed twice with PBS. Following centrifugation at 500 x g for 5 min at 4˚C, cells were resuspended in solution containing Annexin V-FITC and propidium iodide for 15 min at room temperature. Subsequently, the cells were analyzed with a flow cytometer and the FACSCanto™ Plus Software (version 3.0; Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) cellular ROS detection. PC12 cells at 80% confluency were seeded in 6-well plates and stained with 2.5 μM DCFH-DA in 1X dilution buffer provided in the kit (eBioscience; Thermo Fisher Scientific, Inc.) for 20 min at 37˚C; the cells were washed with 1X Buffer three times. Subsequently, cells were harvested with 0.25% trypsin for 5 min at 37˚C and washed with PBS twice, followed by analysis with a flow cytometer for the detection of FITC, using the FACSCanto™ Plus Software (version 3.0; Becton, Dickinson and Company).

JC-1 mitochondrial membrane potential and MitoTracker red assay. PC12 cells were seeded in 6 well plates and labeled according to the manufacturer's protocols (cat. no. ab113850, Abcam). Cells were then cultured for 20 min at 37˚C and washed with 1X dilution buffer provided in the kit (eBioscience; Thermo Fisher Scientific, Inc.) three times. Subsequently, cells were harvested and analyzed with a flow cytometer using the FACSCanto™ Plus Software

Table I. Primers for reverse transcription-quantitative polymerase chain reaction.

Gene name	Forward sequence	Reverse sequence
Mouse *Pah*	5'-TTGTCCTGGAGAACGGAGTC-3'	5'-CTGGATTCAATGTGTGTCAGGTT-3'
Mouse *Got1*	5'-GCGCCTCCATCAGTCTTTG-3'	5'-ATTCATCTGTGCGGTACGCTC-3'
Mouse *Tat*	5'-TGCTGGATGTTCGCGTCAATA-3'	5'-CGGCTTCACCTTCATGTTGTC-3'
Mouse *GAPDH*	5'-CTTCACCACCATGGAGGAGGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'
Rat Pah	5'-ACCCTCTAGGGGTAAATCTTTCA-3'	5'-GAAGCCCCAATGACACAAGC-3'
Rat *Got1*	5'-TCTGCACGTTGCTTGAGTCT-3'	5'-GCCGAGAGACAGAGACGATG-3'
Rat *Tat*	5'-AATGCGGACCTCTGCTATGG-3'	5'-GACAAGCCATCTCCTGTGCT-3'
Rat *GAPDH*	5'-GTGAAGCTCATTTCCTGGTATG-3'	5'-AACTGACGGCCTCTCTCTTG-3'

Got1, aspartate aminotransferase; Pah, phenylalanine hydroxylase; Tat, tyrosine aminotransferase.

(version 3.0; Becton, Dickinson and Company). Fluorescence in FL-1 channel and lacking fluorescence in FL-2 channel is considered to indicate mitochondria with depolarized $\Delta\psi$, suggesting apoptosis or dysfunction (24). As for the MitoTracker labelling assay, PC12 cells were incubated with 200 nM MitoTracker Red (Cell Signaling Technology, Inc.; cat. no. 9082) for 20 min at 37˚C. Following incubation, the cells were rinsed with PBS three times and incubated with regular DMEM culture medium. Subsequently, MitoTracker Red was detected with confocal immunofluorescence analysis using an Olympus FV1000 confocal microscope (Olympus Corporation). In total, five fields per view were imaged (magnification, x400).

2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG) uptake assay. PC12 cells were seeded at $1x10^5$/well in 6-well plate and cultured for 24 h. Then, the cell culture medium was removed and starved for 2 h without glucose and serum. The culture medium was replaced with serum with or without 25 μM 2-NBDG (Sigma-Aldrich; Merck KGaA), and incubated for 40 min at 37˚C. Subsequently, the cells were washed with ice-cold PBS three times and collected for analysis with a flow cytometer with settings for applied for FITC detection.

MTT assay. A total of 3,000 cells/well were cultured with 100 μl DMEM medium in 96-well plate at 37˚C and incubated overnight. Various concentrations of phenylalanine (0, 0.5, 1, 2, 4, 8, 16 and 32 mM) were applied to the cells for 2 or 4 days at 37˚C. Then, 20 μl of 5 mg/ml MTT was added to each well and incubated for 4 h at 37˚C. The medium was removed and 150 μl dimethyl sulfoxide was added; the absorbance at 590 nm was detected with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Statistical analysis. Data are expressed as the mean ± standard error. Statistical analysis was performed using a Student's t-test, one-way analysis of variance followed by a Dunnett's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference. Analyses were performed using Prism (version 7.0; GraphPad Software, Inc., La Jolla, CA, USA). The experiments were performed three times and at least three replicates were present in each group.

Results

Protective effects of HSYA on neurologic behavior and infarction size with TTC staining. As presented in Fig. 1A, an experimental mouse model of cerebral I/R was generated and the protective effect of HSYA was investigated. After 3 days following 2 h of I/R treatment, significant increases in neurological behavior deficits in the I/R group compared with the sham group were observed ($P<0.001$). Of note, administration of 5 mg/kg HSYA notably alleviated the neurological deficits in the I/R + HSYA group compared with the I/R group; however, a significant effect was observed in response to 20 mg/kg HSYA ($P<0.01$; Fig. 1B). TTC staining revealed that the infarct area of the I/R group was ~37% of that of the sham group; however, 5 and 20 mg/kg HSYA significantly decreased the size to 7.2 and 22.4%, respectively ($P<0.05$; Fig. 1C and D). The results indicated that HSYA had an notable protective effect against cerebral I/R injury. To further confirm the neuroprotective effect of HSYA, western blotting was performed to detect the expression of apoptosis and proliferation-associated proteins. The expression of the proliferation markers p-Akt, β-catenin and BCL2 were notably decreased and that of c-Casp3 increased following I/R treatment; however, HSYA rescued the expression of p-Akt, β-catenin and BCL2, and decreased that of c-Casp3 compared with the control (Fig. 1E). In particular, treatment with 20 mg/kg HSYA significantly increased the expression of neuronal cell marker NeuN (Fig. 1E and F), which indicated the neuroprotective effect of HSYA against I/R.

HSYA regulates the expression of enzymes of phenylalanine metabolism to reprogram the metabolic amino acid profile associated with cerebral I/R. To investigate whether I/R and HSYA alter the metabolic profile, plasma samples from the sham and I/R groups treated with or without HSYA were extracted from mouse tail veins and centrifuged at 10,500 x g at room temperature for 5 min for metabolic screening. The results revealed a significant increase in the levels of plasma phenylalanine (from 56.69±1.097 to 95.55±13.01 μmol/l) with I/R stress compared with the sham group Treatment with HSYA significantly reduced the levels of phenylalanine ($P<0.05$; Fig. 2A). The data suggested that HSYA could

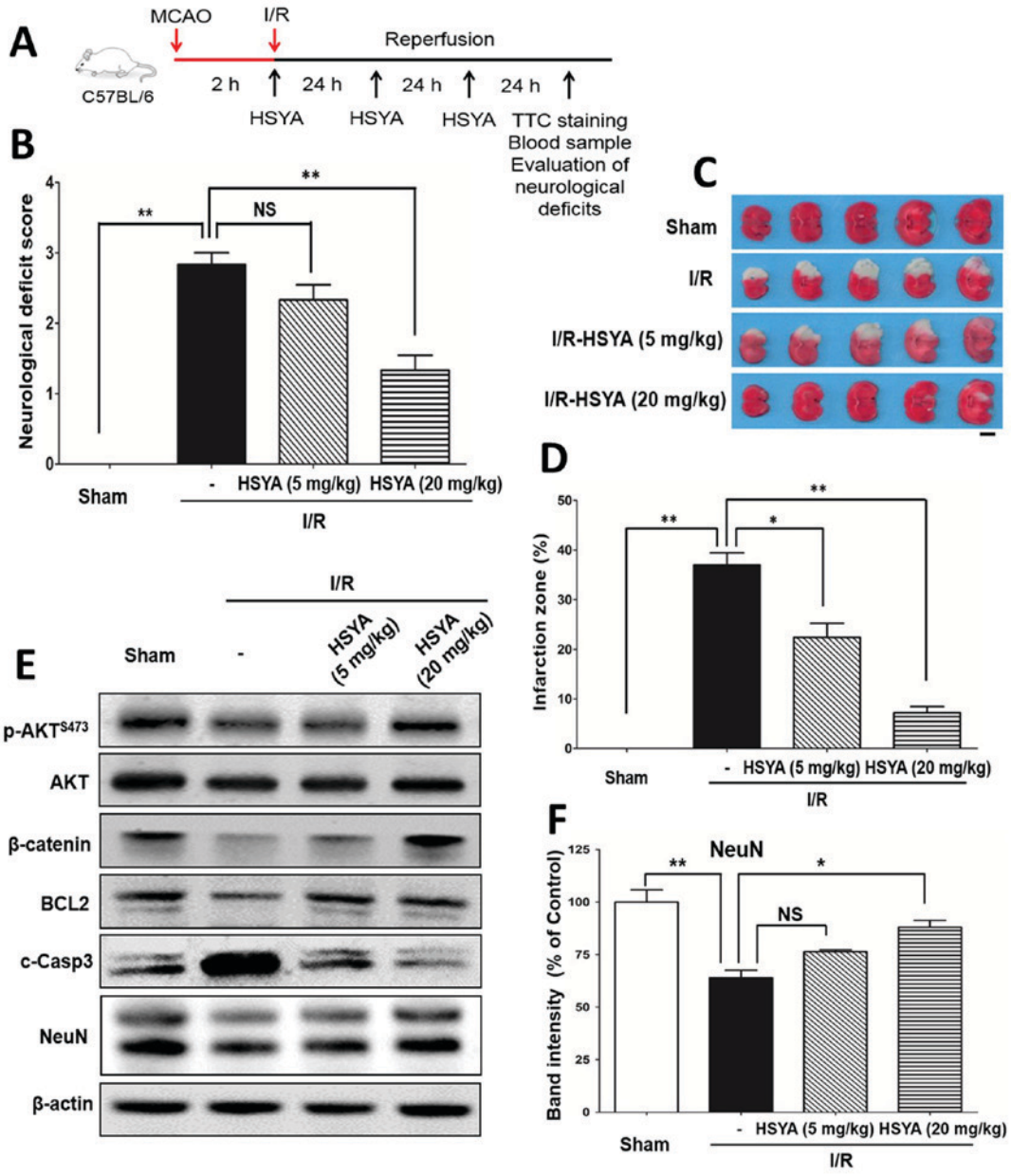

Figure 1. HSYA alleviates neurological deficits and reduces the infarct size induced by cerebral I/R. (A) Experimental scheme for the experimental mice cerebral I/R. Briefly, mice received ischemia via middle cerebral artery occlusion for 2 h and reperfusion for 3 days, with or without HSYA (5 or 20 mg/kg) treatment. (B) Neurological behavior deficit evaluation. A scale of 0-4 points was used to evaluate the neurological deficits. The values are expressed as the mean ± standard error of the mean of 6 mice in each group. I/R significantly increased deficit score, but was reduced following treatment with HSYA. (C) HSYA reduced the infarct zone. TTC staining was used to monitor the infarct area (white). I/R resulted in an infarct zone; HSYA decreased the size of the area. Scale bar=5 mm. (D) Results of statistical analysis of the TTC staining zone with three mice of each group (n=3). (E) Western blotting was conducted to analyze the expression of proliferation and apoptosis-associated molecules with antibodies against p-Akt, β-catenin, BCL2, cleaved caspase-3 and neuron marker NeuN. β-actin was the internal control. (F) Band intensity quantification of NeuN. *P<0.05, **P<0.01. Akt, protein kinase B; BCL2, B-cell lymphoma 2; c-Casp3, cleaved caspase 3; HYSA, hydroxysafflor yellow A; I/R, ischemia/reperfusion; NeuN, neuronal nuclei; p, phosphorylated; TTC, 2, 3, 5-triphenyltetrazolium chloride.

abrogate increases in phenylalanine induced by cerebral I/R. Additionally, whether HSYA affects the expression of key enzymes for the metabolism of phenylalanine was investigated. As presented in Fig. 2B, phenylalanine can be metabolized into tyrosine via PAH; phenylalanine can also be transformed into phenylpyruvate or vice versa via TAT and GOT1. Then, the mRNA expression of these three enzymes were analyzed; significantly decreased *Pah* and *Got1* expression, and increased *Tat* expression were observed in the I/R group compared with the sham group (Fig. 2C-E). Most importantly, HSYA could reverse the altered expression of these three enzymes caused by I/R (Fig. 2C-E). These findings suggested that HSYA may reprogram the metabolism of phenylalanine by regulating the expression of key enzymes.

HSYA inhibits metabolic increases in phenylalanine caused by OGD/R stress in primary mouse neuronal cells. Furthermore, primary mouse neuronal cells were exposed to OGD/R stress to evaluate the protective effects of HSYA. OGD/R treatment was performed as presented in Fig. 3A. Western blotting

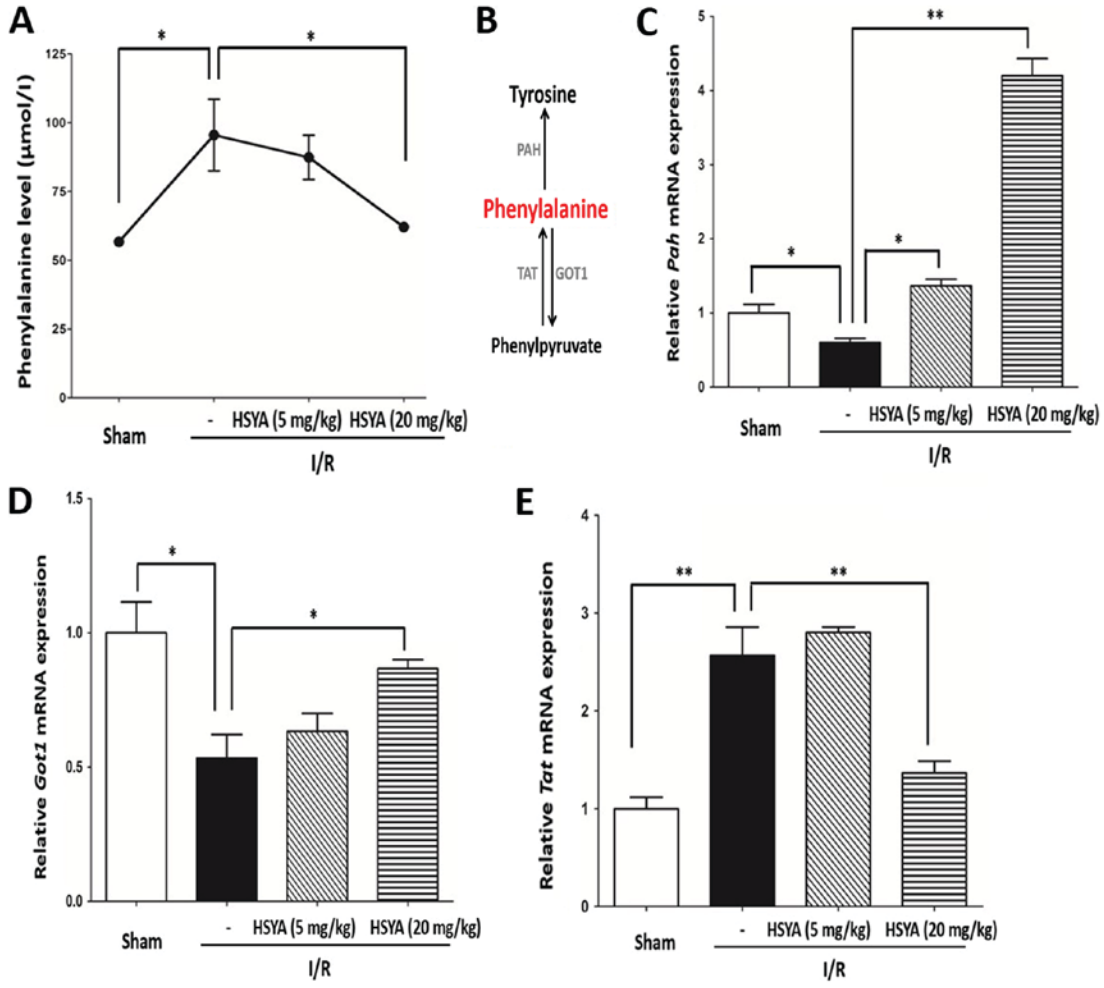

Figure 2. HSYA regulates the levels of phenylalanine and the metabolic enzymes expression caused by cerebral I/R *in vivo*. (A) I/R stress increased the plasma levels of phenylalanine, which was reversed by HSYA. (B) Transformation flux of phenylalanine. Phenylalanine can be metabolized to phenylpyruvate and tyrosine via GOT1 and TAT, and PAH, respectively. HSYA reduced the levels of phenylalanine by regulating the expression of key metabolic enzymes. mRNA expression and relative quantification of (C) *Pah*, (D) *Got1* and (E) *Tat*. The data are expressed as the mean ± standard error of the mean. *P<0.05, **P<0.01. GOT1, aspartate aminotransferase; HYSA, hydroxysafflor yellow A; I/R, ischemia/reperfusion; PAH, phenylalanine hydroxylase; TAT, tyrosine aminotransferase.

demonstrated that HSYA significantly inhibited the expression of c-casp3; the expression of p-Akt, BCL2 and NeuN had recovered in response to HSYA following I/R (Fig. 3B). Additionally, as expected, HSYA could significantly inhibit neuronal cell apoptosis induced by OGD/R treatment; the effect was notably greater in response to 10 μM HSYA than 1 μM (Fig. 3C and D). Therefore, HSYA may protect mouse neuronal cells from OGD/R stress.

To evaluate whether HSYA also alters the metabolic profile associated with OGD/R in primary neurons, metabolic screening was performed. The results revealed alterations in phenylalanine levels, which was similar to the *in vivo* model (I/R injury). HSYA also restricted the increase of phenylalanine in primary neurons with OGD/R. Thus, alterations in the levels of phenylalanine were markedly coincident *in vivo* and *in vitro* (Figs. 2A and 3E). Furthermore, the mRNA expression levels of *Pah, Got*1 and *Tat* in the mouse neuron model was similar to the results of the *in vivo* model (Fig. 3F-H). Therefore, HSYA may recover the levels of phenylalanine by regulating the expression of key metabolic enzymes in a mouse model of I/R and in primary mouse neuronal cells exposed to OGD/R stress. Thus, the neuroprotective properties of HSYA may occur by recovering the levels of phenylalanine dysregulated by I/R injury.

HSYA protects PC12 cells exposed to OGD/R stress via the regulation of phenylalanine metabolism. In addition, to further confirm the neuroprotective effects of HSYA, as previously reported (25), an OGD/R model in PC12 cells was generated (Fig. 4A). Similar to the primary mouse neuronal cells, OGD/R significantly induced the apoptosis of PC12 cells compared with the control; HSYA significantly protected against apoptosis caused by OGD/R stress (P<0.01; Fig. 4B). Of note, the levels of phenylalanine in PC12 cells were significantly increased in response to OGD/R compared with the control, whereas the levels were significantly reduced following treatment with HSYA (P<0.05; Fig. 4C).

Furthermore, alterations in the mRNA expression levels of *Pah, Got1* and *Tat* in PC12 cells were similar within the *in vivo* model and primary mouse neuronal cells (Fig. 4D-F). Therefore, *in vivo*, mouse I/R injury and neuronal cells

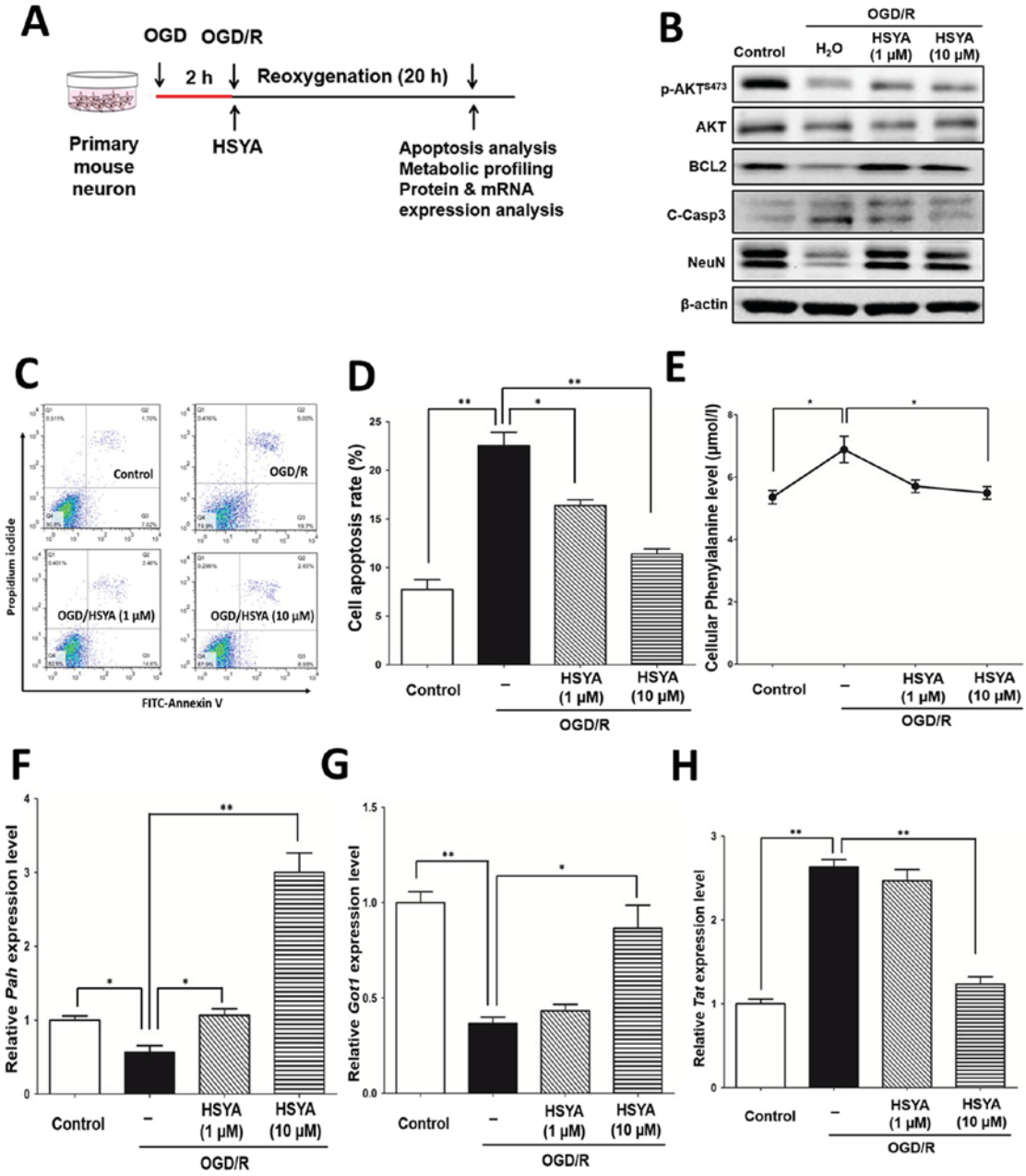

Figure 3. HSYA regulates the levels of phenylalanine in primary mouse neuronal cells with OGD/R stress. (A) Experimental scheme for primary mouse neurons with OGD/R treatment. (B) Analysis of p-Akt, BCL2, cleaved caspase-3 and NeuN levels by means of western blotting in primary mouse neuron with OGD/R treatment. (C) Analysis of apoptosis via flow cytometry. (D) Quantification of apoptosis. (E) Quantification of phenylalanine levels in the control and OGD/R groups treated with or without HSYA. HSYA regulates the expression of key metabolic enzymes in primary mouse neuronal cells. mRNA expression and relative quantification of (F) *Pah*, (G) *Got1* and (H) *Tat*. The data are expressed as the mean ± standard error of the mean. *P<0.05, **P<0.01. Akt, protein kinase B; BCL2, B-cell lymphoma 2; c-Casp3, cleaved caspase 3; GOT1, aspartate aminotransferase; HYSA, hydroxysafflor yellow A; PAH, phenylalanine hydroxylase; NeuN, neuronal nuclei; OGD/R, oxygen and glucose deprivation/reoxygenation; p, phosphorylated; TAT, tyrosine aminotransferase.

in vitro exposed to OGD/R stress exhibited increased levels of phenylalanine by suppressing the expression of key metabolic enzymes, *pah* and *Got1*, and upregulating *Tat* expression (Fig. 4G). HSYA may reduce the levels of phenylalanine by regulating the expression of these three key enzymes (Fig. 4G). Thus, the neuroprotective properties of HSYA may occur by recovering the levels of phenylalanine induced by I/R injury and OGD/R stress (Fig. 4G).

HSYA promotes mitochondrial function and biogenesis associated with its neuroprotective effect against OGD/R stress in PC12 cells. To clarify the neuroprotective mechanism of HSYA, whether HSYA affected the mitochondrial function in PC12 cells was investigated. As presented in Fig. 5A, OGD/R stress significantly increased the ROS levels of PC12 cells compared with the control, as detected by DCFH-DA analysis. As expected, HSYA significantly reduced the ROS levels induced by OGD/R compared with the OGD/R group (P<0.01; Fig. 5A). JC-1 staining revealed that HSYA (10 μM) significantly promoted mitochondrial function by increasing membrane potential suppressed by OGD/R stress (P<0.01; Fig. 5B). Additionally, HSYA significantly enhanced the

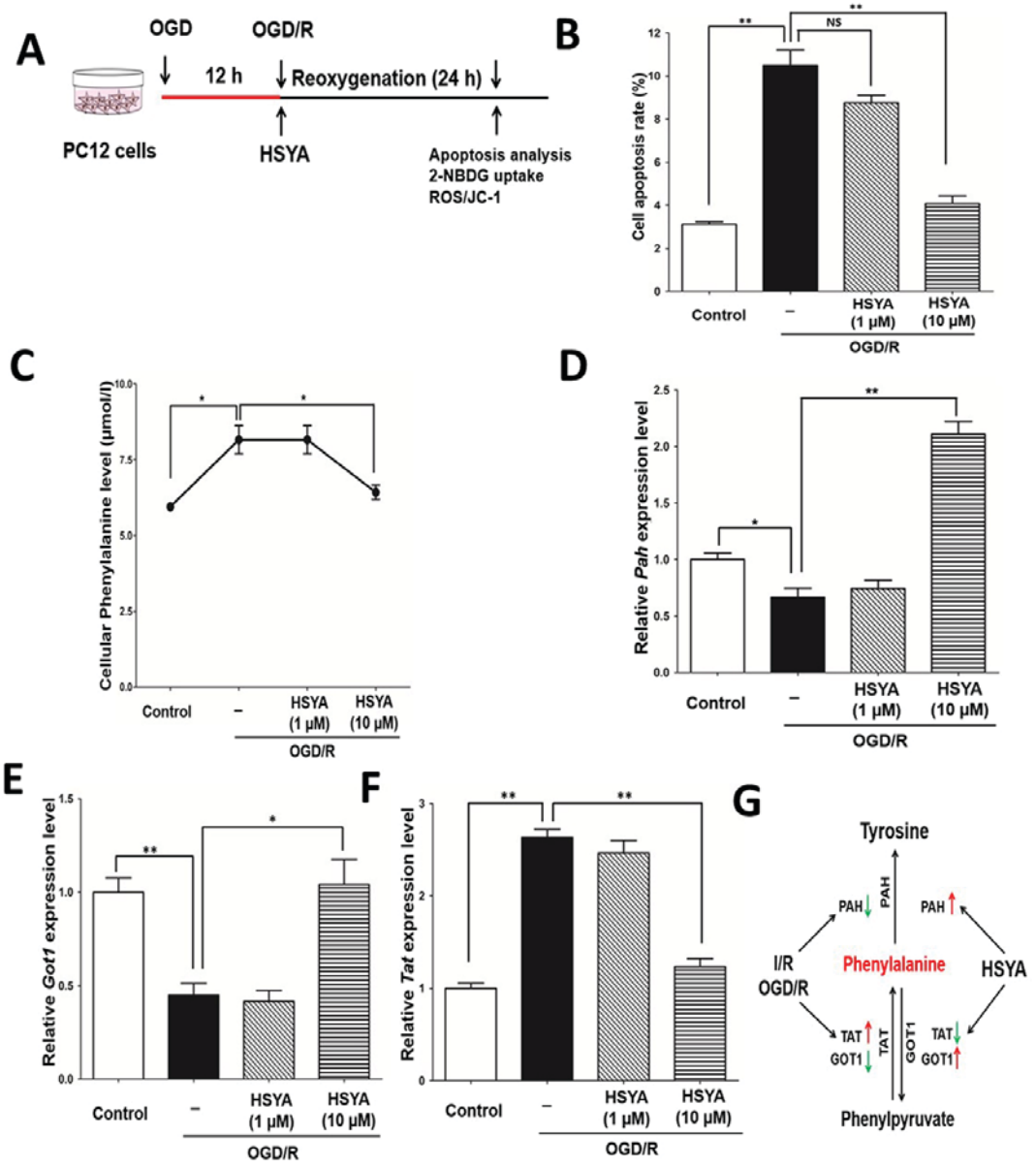

Figure 4. HSYA suppresses the apoptosis and alters the expression of enzymes associated with the metabolism of phenylalanine in PC12 cells with OGD/R stress. (A) Experimental scheme for PC12 cells exposed to OGD/R. (B) Apoptosis analysis and quantification in each group via flow cytometry. (C) Quantification of phenylalanine levels in the control and OGD/R groups treated with or without HSYA. HSYA regulated the expression of key metabolic enzymes of phenylalanine in PC12 cells. Relative quantification of the mRNA expression of (D) *Pah*, (E) *Got1* and (F) *Tat*. (G) Summarization of HSYA regulating metabolic enzymes expression to reduce the levels of phenylalanine. All data are expressed as the mean ± standard error of the mean. *P<0.05, **P<0.01. Got1, aspartate aminotransferase; HYSA, hydroxysafflor yellow A; Pah, phenylalanine hydroxylase; OGD/R, oxygen and glucose deprivation/reoxygenation; p, phosphorylated; Tat, tyrosine aminotransferase.

glucose uptake ability of PC12 cell as determined via 2-NBDG analysis (P<0.01; Fig. 5C).

Furthermore, via MitoTracker red assay, it was demonstrated that mitochondria were small and rod-like following OGD/R (Fig. 5D); however, HSYA promoted the morphological alteration of mitochondria to a narrow and elongated shape, indicating mitochondria fission and biogenesis potential (26). Most importantly, HSYA treatment significantly increased the expression of mitochondria fission protein DRP1 and notably inhibited that of the mitochondria fusion protein OPA1 compared with the OGD/R group (Fig. 5E and F). Additionally, treatment with HSYA increased the protein expression level of VDAC, a mitochondrial marker, suggesting an increase in mitochondrial number (Fig. 5E). These data demonstrated HSYA could promote mitochondria function and biogenesis for its neuroprotective effects in PC12 cells exposed to OGD/R stress.

HSYA recovers the function of mitochondria suppressed by phenylalanine in PC12 cells. As an endogenous metabolite, it is difficult to inhibit the production of phenylalanine. To evaluate whether phenylalanine can directly impair mitochondrial function in neuronal cells, the proliferation of PC12 cells treated with phenylalanine was analyzed. As presented in Fig. 6A, higher concentrations of phenylalanine inhibited

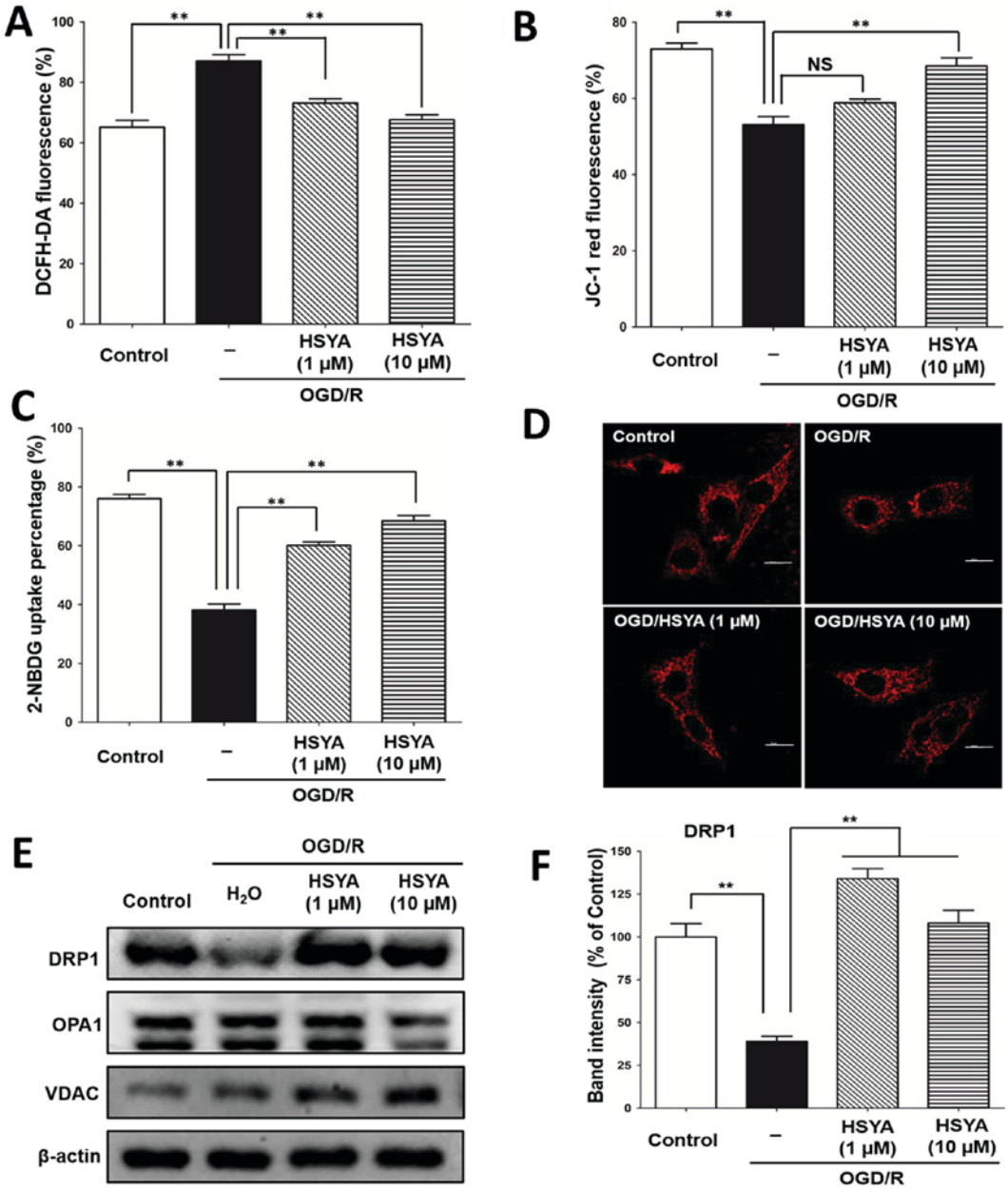

Figure 5. HSYA promotes mitochondrial function and biogenesis in PC12 cells exposed to OGD/R stress. (A) DCFH-DA analysis in PC12 cells with OGD/R stress and HSYA treatment as indicated. (B) JC-1 red fluorescence assay in PC12 cells with OGD/R stress and HSYA treatment. (C) Glucose uptake ability in PC12 cells exposed to OGD/R stress and HSYA as detected by 2-NBDG labelling. (D) MitoTracker Red fluorescence in PC12 cells was detected with a confocal immunofluorescence microscope. Mitochondrial shape was compared in each group. Scale bar=50 μm. (E) Western blotting analysis of mitochondrial fission protein DRP1, fusion protein OPA1 and mitochondrial marker VDAC. β-actin was the internal control. (F) Band intensity quantification of DRP1 in (E). All data are expressed as the mean ± standard error of the mean. **P<0.01. 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose; DCFH-DA, dichloro-dihydro-fluorescein diacetate; DRP1, dynamin-1-like protein; HYSA, hydroxysafflor yellow A; OPA1, dynamin-like GTPase; NS, not significant; VDAC, voltage-dependent anion-selective channel 1; OGD/R, oxygen and glucose deprivation/reoxygenation.

the proliferation of PC12 cells (16 and 32 mM). Analysis of JC-1 red fluorescence demonstrated that phenylalanine significantly inhibited the mitochondrial function in PC12 cells, and HSYA (10 μM) was able to increased mitochondrial function impaired upon treatment with phenylalanine (P<0.05; Fig. 6B). In addition, phenylalanine significantly reduced the expression of the mitochondrial fission protein DRP1 expression compared with the control, which was rescued by HSYA treatment (Fig. 6C and D). Treatment with phenylalanine did not alter the protein expression level of Fis1. However, the protein expression level of MFN2 was slightly increased upon treatment with phenylalanine, although the mechanism underlying MFN2 upregulation remains to be investigated. The findings suggested that HSYA may promote mitochondrial function and biogenesis suppressed by phenylalanine in PC12 cells.

Discussion

Stroke is the second most common cause of mortality worldwide, causing ~6.2 million cases of mortality annually (27). There are two major types of stroke: Ischemic and hemorrhagic.

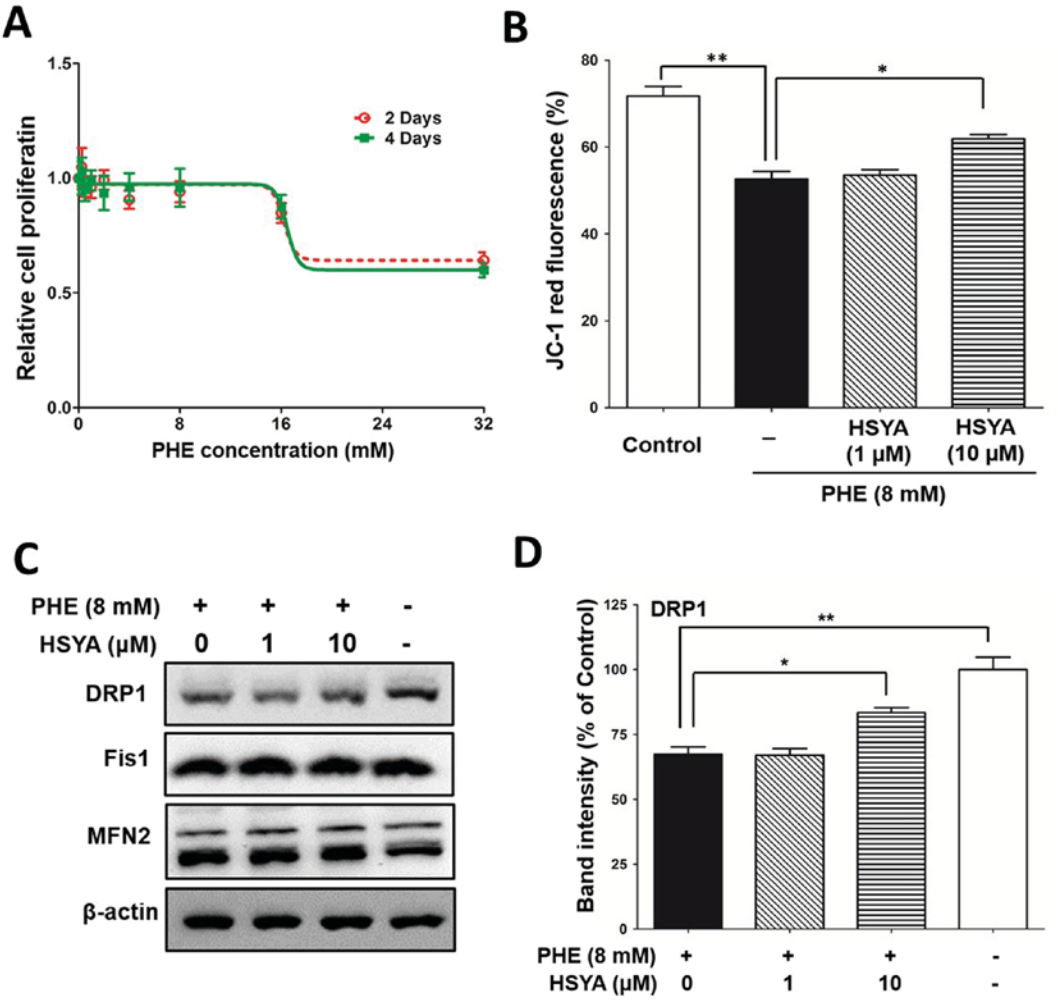

Figure 6. HSYA protects mitochondrial function suppressed by PHE in PC12 cells. (A) MTT assay in PC12 cells with PHE treatment for 2 and 4 days with increased concentration (0, 0.5, 1, 2, 4, 8, 16 and 32 mM). (B) JC-1 red fluorescence assay in PC12 cells with 8 mM PHE and HSYA (1 and 10 μM) treatment as indicated. (C) Western blotting analysis of mitochondrial fission protein DRP1 and Fis1, and fusion protein MFN2. β-actin was the internal control. (D) Band intensity quantification of DRP1. All data are expressed as the mean ± standard error of the mean. *P<0.05, **P<0.01. DRP1, dynamin-1-like protein; Fis1, mitochondrial fission 1 protein; HYSA, hydroxysafflor yellow A; MFN2, mitofusin-2; OGD/R, oxygen and glucose deprivation/reoxygenation; PHE, phenylalanine.

Ischemic stroke, which occurs in ~87% of patients, is mainly caused by an interruption of the blood supply to certain regions of the brain (28). Despite major advances in stroke imaging and treatment, stroke continues to threaten patients and causes familial and societal burden. The initial goal of therapy in ischemic stroke is to restore blood flow. The phenomenon that successful alleviation of regional tissue hypoxia exacerbates reperfusion injury in the form of cell death requires further investigation. Numerous studies using cerebral I/R animal models have identified the mechanism of I/R injury and provided a wide array of neuroprotective strategies to reduce the deleterious effects of reperfusion injury (3); however, the approaches for predicting the efficacy of these strategies are limited.

Several studies reported that the levels of numerous metabolites were altered with cerebral ischemia (16). In particular, branched chain amino acids are notably reduced in ischemic stroke, and the extent correlates with poor neurological outcome (29). In the present study, alterations in the metabolic amino acid profile due to cerebral I/R injury were observed. I/R stress increased the levels of phenylalanine and altered metabolic flux. The findings of the present study demonstrated that I/R and OGD/R stress caused a significant increase in phenylalanine. Furthermore, the significant alterations in the mRNA expression levels of the key metabolic enzymes *Pah*, *Tat* and *Got1* were reported, which are responsible for the metabolism of phenylalanine (18). The underlying mechanism as to how I/R stress regulates the expression of metabolic enzymes remains unknown; however, the findings of the present study indicates that the increased ROS levels induced by I/R injury may be involved.

HSYA has been widely used for the treatment of cerebrovascular diseases in clinical practice. Our previous study revealed that HSYA could reduce ROS levels and activate cellular survival signaling in a myocardial I/R rat model (30). Additionally, the neuroprotective effect of HSYA in a cerebral I/R mouse model was demonstrated in the present study. Furthermore, HSYA reduced the levels of malondialdehyde, and increased those of glutathione and SOD to suppress ROS; thus, apoptosis was inhibited (data not shown). The present study also proposed that HSYA could activate Akt and β-catenin signaling to promote neuronal cell survival.

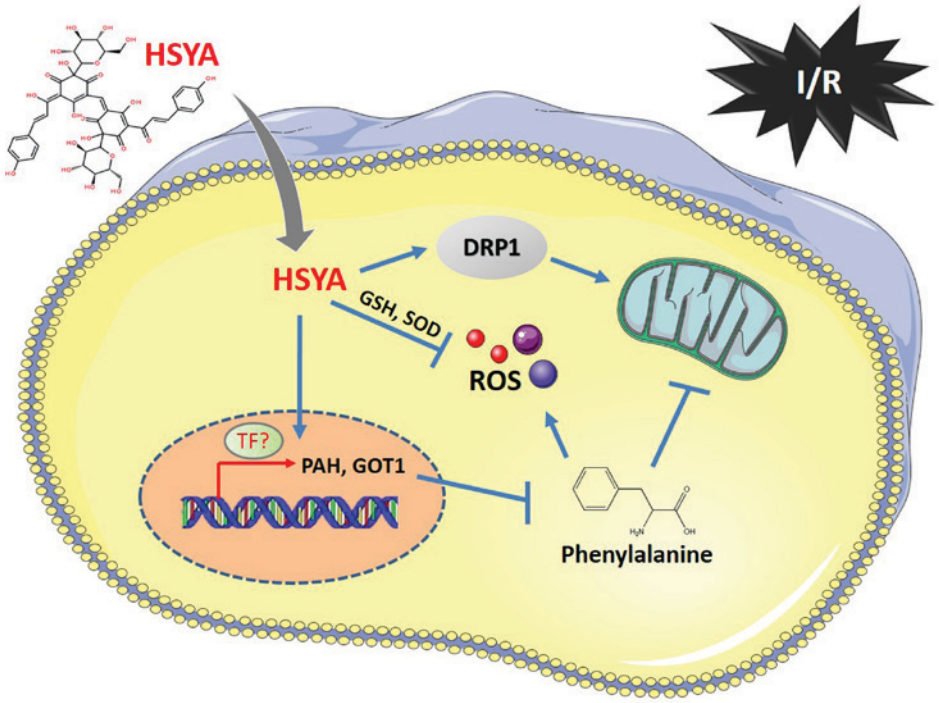

Figure 7. Diagram of HSYA regulating the levels of phenylalanine, mitochondrial function and biogenesis for neuroprotection. I/R injury caused increased ROS and phenylalanine levels to induce neuronal cell apoptosis. HSYA could reduce the production of ROS and phenylalanine by regulating the expression of key metabolic enzymes, including PAH, TAT and GOT1. In addition, HSYA could promote mitochondrial function and biogenesis by upregulating mitochondria fission protein DRP1. GOT1, aspartate aminotransferase; GSH, glutathione; HYSA, hydroxysafflor yellow A; PAH, phenylalanine hydroxylase; ROS, reactive oxygen species; SOD, superoxide dismutase.

Metabolic screening was conducted to identify alterations in the metabolic amino acid profile in the present study. The results revealed that increases in the levels of phenylalanine were induced by cerebral I/R injury. Interestingly, the *in vivo* and *in vitro* models demonstrated that HSYA could reduce alterations in phenylalanine levels caused by I/R and OGD/R stress. Furthermore, we explored the potential mechanism and reported that HSYA could regulate the expression of the key enzymes *Pah*, *Tat* and *Got1*, which are responsible for phenylalanine transformation. Finally, whether phenylalanine can directly impair the function of mitochondria in neuronal cells was determined. Thus, the levels of phenylalanine may be an ideal biomarker for evaluating HSYA as a therapeutic agent.

As a metabolite, phenylalanine possesses a physiological function associated with amino acid metabolism. A previous study demonstrated that phenylalanine may inhibit neurite outgrowth by interfering with L1-mediated cell adhesion (31). The results of the present study revealed that higher concentrations of phenylalanine inhibited the function of mitochondria and cell proliferation; however, phenylalanine alone could not induce apoptosis. Additional conditions, including an increased concentration of phenylalanine or a combination of multiple metabolites, may lead to an increase in apoptosis.

Mitochondria are critical for cellular metabolism and the regulation of apoptosis. The biogenesis of mitochondria is mostly dependent on the process of mitochondrial fission and fusion (32). In the present study, I/R and OGD/R stress increased the levels of phenylalanine, damaged mitochondrial function and induced ROS production (Fig. 7). Whereas, HSYA could reduce phenylalanine levels and promote mitochondrial function via the upregulation of mitochondrial fission protein DRP1. Thus, HSYA may promote mitochondria function and biogenesis in association with its neuroprotective effects. The present study proposed a novel metabolite as a biomarker for cerebral I/R injury and provided a novel mechanism for the development of therapeutic strategies based on HSYA to treat ischemic strokes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AW and XL designed and supervised the experiments. SC and MS conducted the mouse I/R injury, OGD/R model, metabolomics analysis, qPCR, western blotting and the cellular experiments. XZ performed the experiments on primary

neuron cells. ZY, WL and JC performed the cellular reactive oxygen species detection analysis and the cell proliferation assay. YQ performed the statistical analysis. ZY, WL, JC and YQ gave critical comments and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Care Committees of the Fourth Military Medical University (Xi'an, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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