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ORIGINAL ARTICLE

A Novel Plant-Produced Asialo-rhuEPO Protects Brain from Ischemic Damage Without Erythropoietic Action

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Abstract

Mammalian cell-produced recombinant human erythropoietin (rhuEPO^{M}) has been shown to be a multimodal neuroprotectant targeting an array of key pathological mechanisms in experimental stroke models. However, the rhuEPO^{M} clinical trials were terminated due to increased risk of thrombosis, largely ascribed to its erythropoietic function. We recently took advantage of a plant-based expression system lacking sialylation capacity to produce asialo-rhuEPO^{P}, a rhuEPO derivative without sialic acid residues. In the present study, we proved that asialo-rhuEPO^{P} is non-erythropoietic by repeated intravenous injection (44 μg/kg bw) in mice showing no increase in hemoglobin levels and red blood cell counts, and confirmed that it is non-immunogenic by measuring humoral response after immunizing the mice. We demonstrate that it is neuroprotective in a cerebral ischemia and reperfusion (I/R) mouse model, exhibiting ~ 50% reduction in cerebral infarct volume and edema, and significant improvement in neurological deficits and histopathological outcome. Our studies further revealed that asialo-rhuEPO^{P}, like rhuEPO^{M}, displays pleiotropic neuroprotective effects, including restoring I/R-interrupted mitochondrial fission and fusion proteins, preventing I/R injury-induced increase in mitophagy and autophagy markers, and inhibiting apoptosis to benefit nerve cell survival. Most importantly, asialo-rhuEPO^{P} lacking erythropoietic activity and immunogenicity holds great translational potential as a multimodal neuroprotectant for stroke treatment.

Keywords Multimodal neuroprotectant · Asialo-rhuEPO · Cerebral ischemia · Non-erythropoietic · Non-immunogenic · Mitochondrial fission and fusion proteins

Introduction

Stroke is still the leading cause of long-term disability and the fifth leading cause of death in the USA. Acute ischemic stroke (AIS), resulting from the blockage of an artery in the brain, accounts 87% of all stroke cases [1]. Timely reperfusion to re-establish blood flow is the most effective therapy to reduce brain injury and cell death during AIS, even though this process could exacerbate injury through multiple pathophysiological mechanisms [2, 3]. To protect the brain from I/R injuries, various therapeutic strategies have been explored, such as inhibiting apoptosis, promoting angiogenesis, suppressing the inflammation caused by immune response, reducing reactive oxygen species, and regulating the metabolic processes [1, 2, 4]. Clinical studies with tissue plasminogen activator (tPA) and preclinical studies with numerous pharmacological agents including mammalian cell-produced recombinant human erythropoietin (rhuEPO^{M}) have demonstrated that these are protective

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against cerebral I/R injuries [3, 4]. These studies have made remarkable progress in the understanding of brain I/R injury pathophysiology, but no further treatment has been introduced from bench to bedside since tPA was approved by the FDA in 1996 [1, 3]. Because less than 5% of stroke patients are eligible for tPA due to its narrow therapeutic window and multiple contraindications [1, 3], therapeutic options beyond tPA are urgently needed.

Moreover, single specific target therapies were found to be insufficient because ischemic stroke involves multiple pathophysiological mechanisms [5, 6]. Multimodal approaches targeting an array of key mechanisms have been suggested to provide long-term benefits [5–7]. Among tested pharmacological agents, rhuEPO^{M}, a glyco-hormone best known for its regulatory role in the production of red blood cells (RBCs) [8], has been shown to exhibit remarkable neuroprotection in animal models of ischemic stroke [9–11]. Its neuroprotective properties were revealed to be mediated through pleiotropic effects, including anti-oxidative, anti-apoptotic, anti-inflammatory, and anti-excitotoxic as well as angiogenic and neurogenic effects [11–14]. However, its neuroprotective effects from preclinical studies could not be reproduced in clinical trial [15]. Its failure may be ascribed to the adverse effects associated with its hematopoietic activity [10, 14, 16]. For tissue protection, rhuEPO^{M} doses at least five times higher than those required for stimulation of erythropoiesis are needed, and its hematopoietic activity at these high doses can stimulate mass production of RBCs to exacerbate the blood vessel blockade and tissue damage [13]. Its hematopoietic activity-mediated adverse effects are thought to mask or even reverse the tissue-protective effects of rhuEPO^{M} in clinical studies, which compelled the scientific community to look for tissue-protective rhuEPO^{M} derivatives lacking hematopoietic activity.

Several strategies have been employed to develop EPO derivatives that retain tissue-protective function, but lack hematopoietic activity. Short EPO peptides were developed and proved to be tissue-protective and non-erythropoietic [17, 18], but possessed low in vivo stability, high conformational flexibility, and furthermore, the off-target effects of peptide drugs have raised safety concerns [19]. In addition to EPO peptides, asialo-rhuEPO^{E} (enzymatically prepared asialo-rhuEPO) (Fig. 1a) and carbamylated EPO (CEPO) prepared by enzymatic removal of sialic acid residues or carbamylation, respectively, of rhuEPO^{M} as well as mutant EPO (MEPO) made by replacing a single amino acid within the erythropoietic motif were found to be non-erythropoietic but retain tissue protective activity in stroke, sciatic nerve injury, spinal cord compression, and I/R kidney injury [20–23]. Asialo-rhuEPO^{E}, in particular, has been documented to have capacity to cross the blood–brain barrier [20, 24] and exert neuroprotective functions [20, 24–27]. However, only small amounts of asialo-rhuEPO^{E} were prepared by enzymatic desialylation of rhuEPO^{M} for preclinical studies. This approach is not economically viable for large-scale production because of the prohibitive cost [28] and limited production of rhuEPO^{M} [29]. Lack of a suitable expression system and its limited and costly production were major obstacles in the way of exploiting its broad tissue-protective properties and translating its protective effects into clinical practice.

We took advantage of the plant-based expression system naturally lacking sialylation capacity to produce asialo-rhuEPO (designated as asialo-rhuEPO^{P}) in stable transgenic plants by co-expressing human *EPO* and *GalT* (encoding β1,4-galactosyltransferase) genes [30]. Plant-based expression system also offers several advantages, such as free of human pathogens, ease of scaling up production, and cost-favorability [31]. Purified asialo-rhuEPO^{P} from transgenic plant leaves was found to display superior in vitro cytoprotective effects compared with rhuEPO^{M} in protecting neuronal-like mouse neuroblastoma cells [30], pancreatic β-cells [32], and HL-1 cardiomyocytes [33] from staurosporine-induced cell death. These in vitro cytoprotective studies, along with previously reported in vivo neuroprotective effects of asialo-rhuEPO^{E} [20, 24–27], led us to evaluate in vivo neuroprotective properties of asialo-rhuEPO^{P}.

In the present study, we confirm that asialo-rhuEPO^{P} is non-erythropoietic and non-immunogenic. We also demonstrate that it is neuroprotective in a cerebral I/R injury mouse model, and displays its protective effects through regulating multiple cellular pathways, which include promoting cell survival signaling, preventing ischemia-induced mitochondrial fission and fusion protein alterations, inhibiting autophagy and mitophagy markers, and suppressing cell death signaling. These results suggest potential for translation of asialo-rhuEPO^{P} for treatment of stroke as well as a range of neurological diseases including neurodegenerative diseases in which mitochondrial dysfunction occurs [34].

Materials and Methods

Animal Studies

Both male and female BALB/c mice were purchased from Charles River Laboratories, USA. This study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Association and Accreditation of Laboratory Animal Care International guidelines and under the authorization of the Institutional Animal Care and Use Committee at the North Carolina Central University, Durham. Animals were placed on a standard rodent diet ad libitum and housed under a 12-h/12-h light–dark cycle.

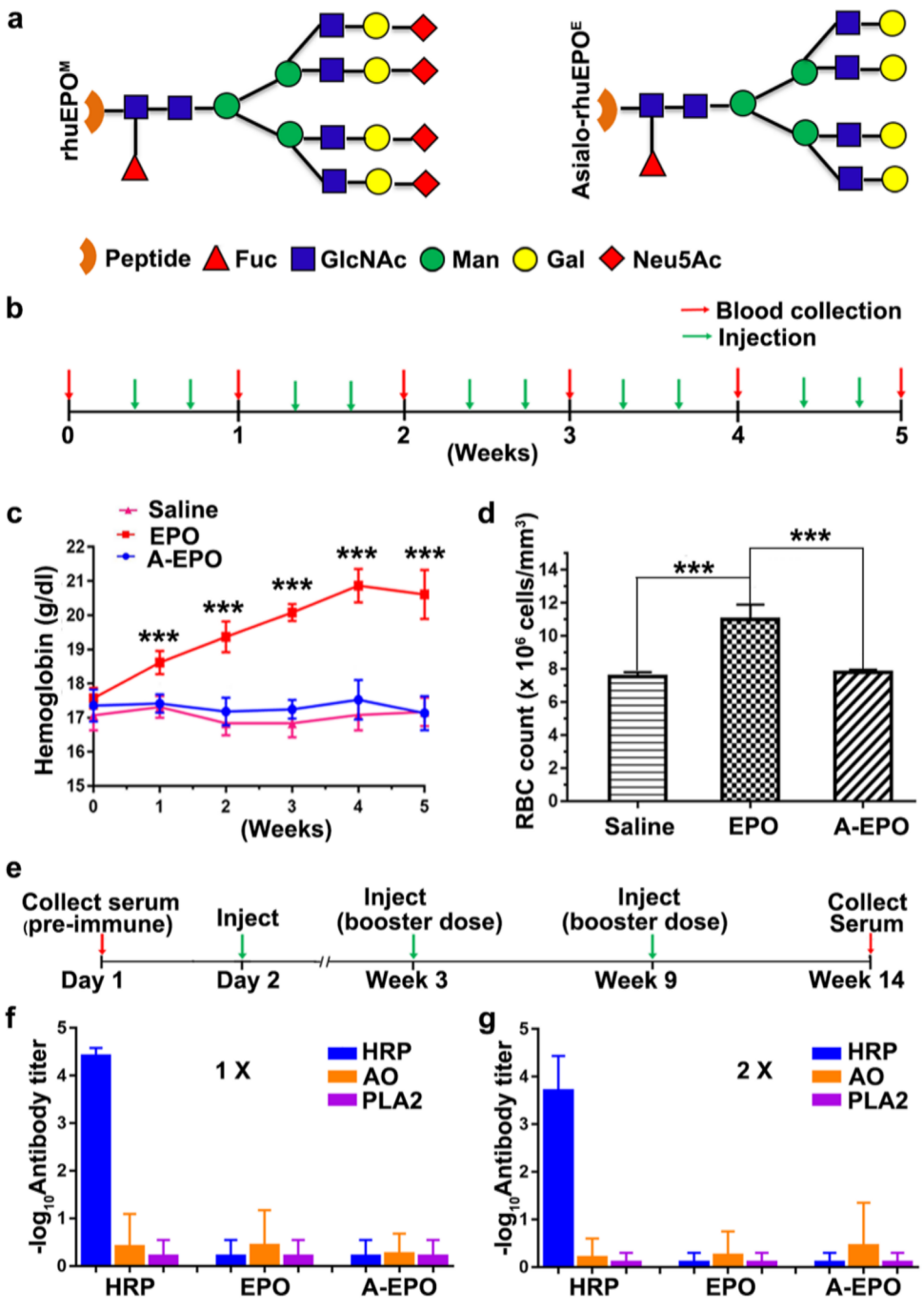


Fig. 1 *N*-Glycan chain, and erythropoietic and immunogenicity of asialo-rhuEPO. **a** *N*-Glycan chains of asialo-rhuEPO^{E} and rhuEPO^{M}. **b** Experimental scheme for testing erythropoietic activity. Asialo-rhuEPO^{P} (A-EPO) or rhuEPO^{M} (EPO) (44 μg/kg bw) was administered intravenously twice/week for 5 weeks. Saline: negative control. **c** The levels of hemoglobin were analyzed weekly. **d** RBC numbers were determined in the fifth week. **e** Experimental scheme for immune response studies. **f**, **g** The antibody titer analysis of sera from BALB/c mice immunized with 1X and 2X neuroprotective dose of A-EPO, HRP (positive control), and EPO (negative control). Humoral response to core α (1,3)-fucose and β (1,2)-xylose on HRP or A-EPO was determined by examining cross-reactivities with two model glycoproteins AO and PLA2 carrying above unique sugars. Green arrows represent injection times while red arrows represent blood collection times. Data plotted are the average ± SD. ***: $p < 0.001$

Confirming Lack of In Vivo Hematopoietic Activity of Asialo-rhuEPO^{P}

Following the previously published protocol [20], female BALB/c mice of age 6 weeks were used for studying hematopoietic activity. Mice were randomly divided into three groups: group 1, received saline (negative control); group 2, received asialo-rhuEPO^{P}; and group 3, received rhuEPO^{M} (positive control) ($n = 8$). Laboratory Rodent Diet 5001 (LabDiet) was provided to animals without additional iron supplemented. Hemoglobin responses to repeated doses of asialo-rhuEPO^{P} versus rhuEPO^{M} were assessed by intravenous administration of 44 μg/kg bw of asialo-rhuEPO^{P} or rhuEPO^{M} or vehicle (saline) twice per week for 5 weeks as previously described [20]. Hemoglobin concentrations were determined with a hemoglobinometer using blood (< 20 μl) withdrawn from the facial vein once per week under isoflurane anesthesia up to 5 weeks. At the end of 5 weeks, RBC counts were measured using a hemocytometer.

Determination of Immune Response to Plant-Specific Sugars on Asialo-rhuEPO^P

Two separate experiments were performed to investigate immune response to plant-specific glycan epitopes (core β (1,2)-xylose and α (1,3)-fucose) on asialo-rhuEPO^P. Horseradish peroxidase (HRP) carrying the same glycan epitopes was used as a positive control whereas rhuEPO^M lacking them was employed as a negative control. Female BALB/c mice were used for this part because they generally have stronger innate and adaptive immune responses than the males [35]. In each experiment, mice of 6 weeks of age were randomly divided into three groups: group 1, immunized with asialo-rhuEPO^P suspended in Freund's incomplete adjuvant; group 2, immunized with HRP with Freund's incomplete adjuvant; and group 3, immunized with rhuEPO^M with incomplete adjuvant. In experiment 1, eight mice per group were immunized subcutaneously with 1X neuroprotective dose (44 μg/kg BW) [20] of asialo-rhuEPO^P, rhuEPO^M, or HRP. In experiment 2, seven mice each of same age and gender were immunized with 2X neuroprotective dose (88 μg/kg BW) of above indicated proteins. In each experiment, two additional booster doses were given after 3 and 9 weeks following the first immunization. Preimmune blood was collected from each mouse to isolate serum before the initiation of immunization. Blood then from each mouse was collected 5 weeks after the last boost to isolate serum. The serum was isolated by centrifugation at 1500 × g for 15 min and antibody titers were determined using ELISA as described previously [36] and detailed in Online Resource 1.

Mouse Model of I/R Injury, Drug Administration, and Determination of Infarct Volumes

The in vivo neuroprotective effects were evaluated in mouse model of transient (1 h) middle cerebral artery occlusion (MCAO) with intravenous administration of single dose of asialo-rhuEPO^P (44 μg/kg bw) at the start of reperfusion. Male BALB/c mice of age 6–8 weeks were selected for this experiment because they have more uniform physiological conditions compared to the female whose menstruation may affect the results. The experiment was conducted with six groups: sham (received midline incision but no occlusion, and saline); sham-rhuEPO^M (sham-operated and received rhuEPO^M); sham-asialo-rhuEPO^P (sham-operated and administered asialo-rhuEPO^P); I/R-saline (animals underwent 1 h MCAO and received saline); I/R-rhuEPO^M (underwent MCAO and administered rhuEPO^M); and I/R-asialo-rhuEPO^P (underwent MCAO and administered asialo-rhuEPO^P). Four mice each were used for sham and sham plus asialo-rhuEPO^P or rhuEPO^M treatment groups while six mice each for I/R-saline and I/R plus asialo-rhuEPO^P or rhuEPO^M treatment groups. The procedures of animal anesthesia, and MCAO and reperfusion surgeries were the same as our previously described [37]. Briefly, a small incision was made on the CCA and a filament (Doccol corporation, USA), which had a distal cylinder of silicon rubber with a diameter of 0.21 ± 0.02 mm, was inserted into the ICA until a faint resistance was felt. After 1-h occlusion, the filament was withdrawn to achieve recirculation. The mice were subjected to a neurological examination immediately after the animals recovered from anesthesia to judge the successful induction of MCAO model using a Zea-Longa 0–4 score scale [38]. The animals with the neurological deficit scores of 2 and higher were selected as the successful MCAO model. MCAO animals were randomly assigned to the control and treated groups. After 24-h reperfusion, the mice were subjected to a neurological examination again to compare the neurological deficits between the experiential groups. After that, mice were sacrificed and whole brains were dissected coronally into 1-mm brain slices using a stainless brain matrix for 2,3,5-triphenyltetrazolium chloride (TTC) test. All the procedures for the TTC test and the measurement of infarct and edema volumes are detailed in Online Resource 2.

Histology and HE, Nissl, and TUNEL Staining

The isolated brains were immersed in 4% paraformaldehyde for 24 h. Four mice were used for each group. The brain tissues were dehydrated and embedded in paraffin. The brain tissue blocks were sectioned at a thickness of 4 μm using a microtome as described previously [39]. The slides containing paraffin sections were placed in a slide holder and used for hematoxylin and eosin (HE), Nissl, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. All these staining procedures are detailed in Online Resource 3. For histology study as well as for following immunocytochemistry and immunoblotting analyses, the fronto-parietal cortex of the right hemisphere (ipsilateral) was used.

Immunofluorescence Assay

For the immunofluorescence assay, sections (4-μm thickness) were deparaffinized, rehydrated, and submerged in citrate buffer (pH = 6). Then they were heated at boiling temperature in a pressure cooker for 10 min for antigen retrieval. The levels of following proteins were examined in each group after an incubation with primary antibody overnight at 4 °C, and then a second incubation with secondary goat anti-rabbit antibody (1:400, ThermoFisher) at 37 °C for 45 min. The information of all primary antibodies and their dilution factors used is detailed in Online Resource 4. DAPI was used to stain the nuclei. The sections were then viewed

under a fluorescence microscope (Olympus). Images were captured at a magnification of 400×.

Western Blot Analysis

For Western blotting, total proteins were extracted from right hemisphere brain tissues of each mouse using N-PER™ Neuronal Protein Extraction Reagent (Cat. No.87792) containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Three mice each were used for each group. Protein concentrations of the samples were measured using a BCA Protein Assay kit (Thermo Fisher Scientific). An equal amount of protein lysates (20 µg) for sample was separated on 4–12% Bis–Tris NuPAGE gels (Invitrogen, Carlsbad, CA, USA) and transferred to PVDF membranes (MilliporeSigma) using a XCell SureLock mini-cell system (Invitrogen). After transfer, membranes were blocked in a 1:1 solution of Li-COR Odyssey Blocking buffer (Li-COR, Inc.) and PBS. The membranes were subsequently probed with primary antibodies. All primary antibodies and their dilution factors used are detailed in Online Resource 4. Following incubation with primary antibodies, the blots then were incubated with secondary antibodies (goat anti-rabbit IgG conjugated to IRDye 680RD and goat-anti-mouse IgG conjugated to IRDye 800CW at 1:10,000 dilution, LI-COR Biosciences). Image acquisition was performed with Li-COR Odyssey Classic Imaging System (Li-COR Inc.). The fluorescent signals were quantified and analyzed with the Li-COR image studio software version 5.2.5, and the target protein signals among samples were normalized to each loading control. Beta-actin detected by anti-beta-actin antibody (A2228, Sigma-Aldrich) was used as an internal control.

Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics 20.0. All values were expressed as mean values ± standard deviation (SD), or as a percentage of the control. Each parameter in all data sets was compared by ANOVA followed by the LSD post hoc test. Statistical significance was set at $p < 0.05$.

Results

Asialo-rhuEPO^{P} Is Non-erythropoietic and Non-immunogenic

Currently, rhuEPO^{M} is being used in clinical practice to treat anemia in patients suffering from chronic kidney failure or after chemotherapy [40]. Typically, a dose of 100 IU/kg (~0.8 µg/kg) bw of EPO protein is used to stimulate RBC production in those patients [41]. For neuroprotection, however, much larger doses are required. A minimum effective rhuEPO^{M} dose of ~4 µg/kg bw has been reported in rodent stroke models [13] and 4–36 µg/kg bw were used in clinical trials [42]. At these high doses, rhuEPO^{M} can stimulate mass production of RBCs and increase the risk of thrombosis [13, 14, 16]. High doses of any EPO derivatives used for tissue-protection can also increase RBC production if they possess erythropoietic activity. Therefore, the effects of repeated injection of asialo-rhuEPO^{P} on hemoglobin concentration and RBC production were evaluated at a neuroprotective dose (44 µg/kg bw) reported previously for asialo-rhuEPO^{E} [20]. If asialo-rhuEPO^{P} possesses any erythropoietic activity, it would become apparent at this dose because it is about 55 times higher than the dose of rhuEPO^{M} typically used for stimulation of erythropoiesis [40]. Intravenous injection of asialo-rhuEPO^{P} twice-a-week for 5 weeks (total of 10 injections) to female BALB/c mice (Fig. 1b) caused no increase in hemoglobin concentrations whereas rhuEPO^{M} increased hemoglobin concentration significantly as early as a week after two injections compared to saline group (Fig. 1c). Consistent with these observations, at the end of 5-week period, the RBC count in mice receiving asialo-rhuEPO^{P} was $7.8 \times 10^{6}/mm^{3}$, similar to saline group ($7.5 \times 10^{6}/mm^{3}$), while that in mice receiving rhuEPO^{M} was $11 \times 10^{6}/mm^{3}$ corresponding to ~47% increase (Fig. 1d). These results confirmed that asialo-rhuEPO^{P} is non-erythropoietic at the neuroprotective dose tested.

Next, we tested immunogenicity of asialo-rhuEPO^{P} because it carries plant-specific core α (1,3)-fucose and β (1,2)-xylose residues [30, 43], which are absent in mammalian glycoproteins. There is an ongoing debate on whether these plant-specific glycans on therapeutic glycoproteins are immunogenic with an increased risk for adverse effects [44–46]. Therefore, we investigated immunogenicity of plant-specific glycan epitopes on asialo-rhuEPO^{P} by immunizing BALB/c mice with 1X (44 µg/kg bw) and 2X (88 µg/kg bw) reported neuroprotective dose [20] of asialo-rhuEPO^{P} along with the same doses of HRP (positive control) and rhuEPO^{M} (negative control) (Fig. 1e). The humoral response to plant-specific glycans was determined as described in the "Materials and Methods" section. The results are expressed as $\log_{10}$ Ab titers in Fig. 1f and 1g after deducting the values obtained for the pre-immune sera. As can be seen from Fig. 1f and 1g with 1X and 2X neuroprotective doses, HRP immunized mice developed a strong humoral response against protein backbone, but not against core β (1,2)-xylose and core α (1,3)-fucose residues, as evident from lack of cross-reactivities of sera with cucumber ascorbate oxidase (AO) and honeybee venom phospholipase A2 (PLA2), which also bear same plant-specific glycans as HRP. In the case of mice immunized with asialo-rhuEPO^{P}, no cross-reactivity with HRP, AO, or PLA2 was observed,

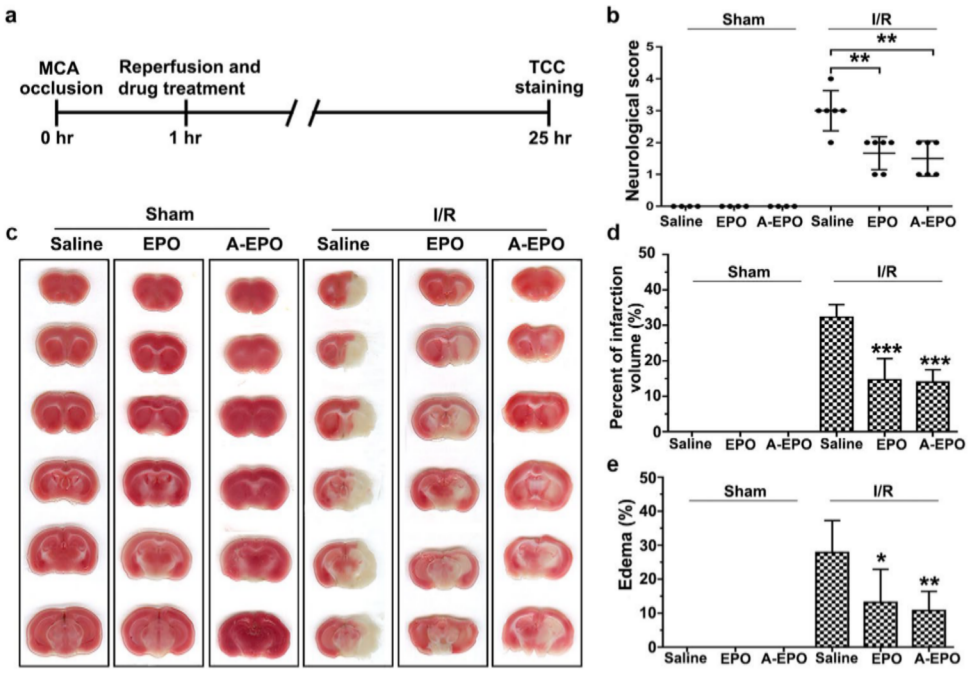


Fig. 2 Neuroprotective effects of asialo-rhuEPO^{P}in a cerebral I/R injury mouse model. **a** Experimental scheme of I/R injury. The experiment was performed under 1 h MCAO and 24 h of reperfusion with six treatments. Drug or saline was administered intravenously at the start of reperfusion. SH: sham; saline (negative control); EPO: rhuEPO^{M} (positive control); A-EPO: asialo-rhuEPO^{P}. **b** Neurological deficit score was evaluated after 24 h of reperfusion. **c** Representative TTC staining results of the brain sections from different treatments. **d** Percentage of cerebral infarction volume. **e** Percentage of edema. Plotted data are the average ± SD. *, **, ***: $p < 0.05$, 0.01, 0.001, A-EPO or EPO versus saline mice under I/R experimental conditions

indicating no humoral response against glycan epitopes even at double the neuroprotective dose. Its $\log_{10}$ Ab titers values were like those of negative control rhuEPO^{M} (Fig. 1f, g). The above results show that asialo-rhuEPO^{P} is non-immunogenic even at high dose, suggesting that it is safe to be used in clinical practice.

Asialo-rhuEPO^{P} Displays Excellent In Vivo Neuroprotective Effects

The neuroprotective effects of asialo-rhuEPO^{P} were evaluated using a mouse model of I/R (1-h occlusion/24-h reperfusion) injury (Fig. 2a). Neurological deficits, infarct, and edema volumes following I/R-injury and asialo-rhuEPO^{P} or rhuEPO^{M} treatments were determined. Intravenous administration of asialo-rhuEPO^{P} or rhuEPO^{M} immediately at the restoration of blood flow showed significant decrease in neurological deficits from 3.1 in I/R-saline group to 1.9 and 1.8, respectively (Fig. 2b). Asialo-rhuEPO^{P} treatment showed significant reduction in cerebral infarction volume from 33.2% in I/R-saline group to 15.4%, and was the same as that of rhuEPO^{M} treatment (15.4%) as revealed by TTC staining (Fig. 2c, d). Moreover, both EPO treatments also significantly reduced edema volume from ~ 30 to ~ 14% (Fig. 2e). When asialo-rhuEPO^{P} or rhuEPO^{M} treatment in sham group compared with vehicle control (sham-saline group), neither of them showed any adverse effects (Fig. 2c–e). Therefore, both sham-asialo-rhuEPO^{P} and sham-rhuEPO^{M} treatments were excluded in the following studies.

Neuroprotective effects of asialo-rhuEPO^{P} were further validated by performing histological and immunofluorescence analyses on brain sections. HE staining of brain sections from sham group showed normal cells (Fig. 3a) with round, lightly stained nuclei whereas sections from I/R-saline group displayed high number of damaged cells with pykanotic nuclei in the cerebral infarction area (Fig. 3a, b). However, brain sections from both asialo-rhuEPO^{P}- and rhuEPO^{M}-treated animals showed significantly decreased

cellular damage as evident from lesser cells with pyknotic nuclei than the non-treated I/R control (Fig. 3a, b). Furthermore, Nissl staining, which is used to stain the Nissl body and nuclei of neurons, revealed remarkably decreased in the Nissl bodies in neurons of the I/R-saline group when compared with the sham group, indicating I/R-induced neuronal cell damage and death (Fig. 3a, c). Brain sections from the asialo-rhuEPO^{P}- and rhuEPO^{M}-treated groups, however, showed higher neuron density and lesser shrinking nuclei than I/R-saline group, indicating that asialo-rhuEPO^{P} and rhuEPO^{M} as well protected neurons from I/R injury (Fig. 3a, c). The neuroprotective effects of asialo-rhuEPO^{P} were further confirmed by Western blotting and immunostaining with anti-NeuN antibody. NeuN is a nuclear protein present in most of the neurons in the central nervous system and has been used as a neuronal marker to assess the functional state of neurons [47]. Western blotting analysis showed dramatically lower NeuN levels in the I/R-saline group (decreased by 75.6%) compared with the sham group while both asialo-rhuEPO^{P} and rhuEPO^{M} treatments were able to significantly restore affected levels (Fig. 3d, e). This was further supported by NeuN-staining of brain sections, which showed remarkably decreased number of NeuN-positive cells in IR-saline group compared to the sham group while asialo-rhuEPO^{P} and rhuEPO^{M} treatments showed lesser NeuN-positive cell loss (Fig. 3f). These results indicate that asialo-rhuEPO^{P} and rhuEPO^{M} as well prevent I/R injury-induced degeneration of neurons.

Asialo-rhuEPO^{P} Regulates Cell Survival Pathways

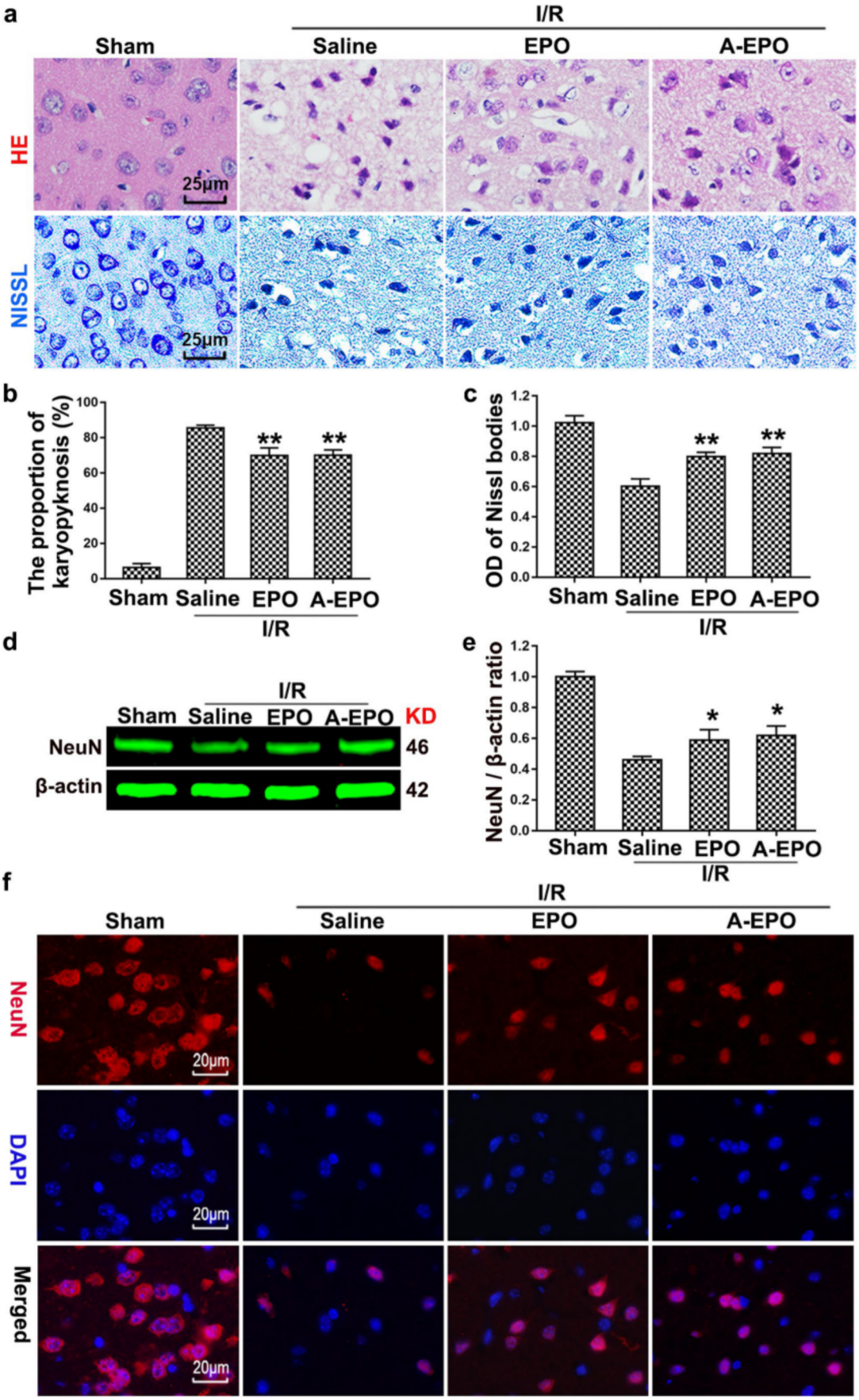
Previous studies have shown that EPO is antiapoptotic and mediates tissue protection by the activating STAT5, PI3K/AKT, and/or MAPK/ERK1 signaling pathways depending on types of tissue and injury, and time of its administration leading to attenuation of apoptosis [12, 13]. Therefore, we investigated which of three survival pathways is activated by asialo-rhuEPO^{P} to display its neuroprotective effects. Western blotting of total protein extracts made from mice brain tissues showed significant increase in phosphorylation of STAT5, PI3K, AKT, and ERK1/2 in asialo-rhuEPO^{P}- and rhuEPO^{M}-treated groups compared with I/R-saline group (Fig. 4a–e). These results revealed that the neuroprotective effects of both EPOs are potentially mediated through activation of STAT5, PI3K/AKT, and MAPK/ERK1/2 signaling pathways, suggesting that asialo-rhuEPO^{P} like rhuEPO^{M} also exhibits pleiotropic effects through these pathways.

Asialo-rhuEPOP Restores I/R-Interrupted Mitochondrial Fission–Fusion–Related Proteins

Previous studies have shown that mitochondria are both a source and target of I/R injury and cell death, and that their dysfunction resulting from the imbalance of mitochondrial fission and fusion is one of the hallmarks of I/R-induced neuronal death [48–51]. Reported anti-oxidative, anti-apoptotic, anti-inflammatory, and anti-excitotoxic effects of EPO on cerebral I/R injury were found to be directly linked to mitochondria's integrity and function through controlling mitochondrial membrane potential, Ca^{2+} buffering, ROS production, and neurotransmission [12–14]. However, whether EPO and its derivatives can regulate mitochondrial fusion and fission to display these pleiotropic protective effects is unknown. Mitochondrial fission and fusion balance is key to maintain mitochondria structure and function for neuronal survival and function [48, 49]. Fission regulates the amounts of mitochondria and removes damaged mitochondria, whereas fusion maintains normal mitochondrial activity by complementation of damaged mitochondrial contents with the components of healthy mitochondria [48, 51]. Both processes are particularly important in neurons that require continuous redistribution of mitochondria across long distances to meet end-to-end energy requirements, Ca^{2+} buffering, and neurotransmission [48]. I/R and other cerebral insults promote fission leading to disturbed mitochondrial dynamics and compromised mitochondrial functions thereby promoting pro-apoptotic factors release, such as cytochrome *c* (cyt *c*) [48, 50, 52]. We therefore analyzed key mitochondrial fission- and fusion-related proteins by Western blotting and immunofluorescence analysis.

First, we investigated important mitochondrial fission-related proteins. Mitochondrial fission in mammals is controlled by dynamin-related protein 1 (Drp1) and Drp1 receptor fission 1 protein (Fis1). Drp1 assembles and oligomerizes on the mitochondrial outer membrane (MOM) to induce fission while Fis1 promotes fission by binding and recruiting Drp1 to the mitochondrial surface [48]. Our Western blotting analysis showed significant increase in the levels of p-Drp1 and Fis1 in the brain tissues from I/R-saline group compared to sham group (Fig. 5a–c), indicating that I/R processes promoted mitochondrial fission. Brain tissues from asialo-rhuEPO^{P}- as well as rhuEPO^{M}-treated mice, however, showed significant reduction in p-Drp1 and Fis1 levels compared to those in I/R-saline group (Fig. 5a–c). The abilities of asialo-rhuEPO^{P} and rhuEPO^{M} treatments to restore I/R injury-induced increase in p-Drp1 and Fis1 levels were further confirmed by immunofluorescence assay, which showed decrease levels of both proteins (Fig. 5d). The above results suggest that asialo-rhuEPO^{P} and rhuEPO^{M} as well suppressed I/R-induced mitochondrial fission.

Next, we determined the levels of key mitochondrial fusion-related proteins across all four groups. The core mitochondrial fusion machinery in mammals is constituted by three GTPases: mitofusins 1 and 2 (Mfn1 and Mfn2) located in the MOM, and optic atrophy protein 1 (OPA1) found in the inner mitochondrial membrane (IMM) [48,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d
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EPO
A-EPO
KD
NeuN
46
β-actin
42
e
NeuN / β-actin ratio
1.2
1.0
0.8
0.6
0.4
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EPO
A-EPO
I/R
f
I/R
Sham
Saline
EPO
A-EPO
NeuN
DAPI
Merged
20μm
20μm
20μm

◂**Fig. 3** The effects of asialo-rhuEPO^{P}on I/R injury-induced histopathological changes. **a** Representative images of the brain sections from different treatments by HE and Nissl staining. **b** Quantitative summary of pyknotic cells from HE staining. **c** OD of Nissl bodies. **d** Representative Western blotting of NeuN. β-Actin was used as an internal control. **e** The ratio between NeuN and β-actin. **f** Immunostaining assay results of NeuN. Brain section was probed with anti-NeuN antibody (red) and stained by DAPI (blue) to observe nuclei. Data plotted are the average ± SD. *, **: $p < 0.05$, 0.01, asialo-rhuEPO^P (A-EPO) or rhuEPO^M (EPO) versus saline mice under I/R experimental conditions

51]. Mfn1 and Mfn2 participate in the fusion of MOM of mitochondria while OPA1 is important for IMM fusion [48, 51]. Western blotting results showed significant decrease in the levels of Mfn2, Opa1, and Mfn1 in the I/R-saline group compared with sham group (Fig. 6a–d), indicating that I/R suppressed mitochondrial fusion. When these three proteins were analyzed in brain tissues from asialo-rhuEPO^P- and rhuEPO^M-treated mice, their affected expression levels following I/R injury were found to be significantly restored (Fig. 6a–d). We further performed immunofluorescence assay on Mfn2, Opa1, and Mfn1, and found that immunofluorescence results well supported obtained Western blotting results (Fig. 6e–g). These results suggest that both types of EPO can restore I/R-interrupted mitochondrial fission–fusion–related proteins to possibly re-balance mitochondrial dynamics.

Asialo-rhuEPO^P Suppresses I/R-Induced Excessive Mitophagy and Autophagy

Mitochondrial fission is followed by mitophagy to clear damaged organelles [53, 54]. Since we discovered that asialo-rhuEPO^P treatment can restore dysregulated mitochondrial fission, we were interested to find out whether asialo-rhuEPO^P treatment could also regulate mitophagy-related proteins. In mammalian cells, the PINK1 (PTEN-induced putative kinase protein 1) and parkin (a E3 ubiquitin ligase) cooperatively sense cellular stress and mediate the removal of damaged mitochondria [53, 54]. Moreover, autophagy has been found to be activated after ischemic stroke [55], and LC3, p62, and Beclin1 are common autophagy markers [56–58]. LC3 is an ubiquitin-like protein with multi-isoforms, which undergoes conjugation with phosphatidylethanolamine to form LC3-II from unconjugated LC3-I upon induction of autophagy [56]. Only LC3B levels have been shown to correlate with the levels of autophagic activity [57]. Protein p62 is a key substrate of autophagy [57] while Beclin1 is part of an autophagy-specific class III PI3K complex, which play important roles in autophagosome formation and autolysosome fusion [58]. Therefore, these markers for mitophagy and general autophagy were analyzed.

Our Western blotting results showed increased levels of PINK1 and parkin in the I/R-saline group (Fig. 7a–c) compared with sham group, consistent with the previous reports of increase in the mitophagy in order to remove damaged mitochondria following I/R injury [53, 54]. In I/R-asialo-rhuEPO^P and I/R-rhuEPO^M groups, PINK1 and parkin levels were significantly lower compared to I/R-saline group, implying that asialo-rhuEPO^P- and rhuEPO^M-treated tissues may have lesser mitophagic activity than I/R-saline group tissues, consistent with lower mitochondrial fission anticipated from restoration of fission-related proteins in these groups (Fig. 5a–c). When general autophagy-related markers LC3B, p62, and Beclin1 were studied, total LC3B (including LC3B II + I), LC3B II/I ratio, and Beclin1 levels were found to be elevated whereas p62 was reduced in I/R-saline group (Fig. 7a and 7d–g) compared with sham group, suggesting that I/R injury also induces autophagy as reported previously [55]. Like mitophagy, I/R injury-induced excessive autophagy could be restored by asialo-rhuEPO^P and rhuEPO^M treatments as evidenced by bringing expression levels of all tested proteins close to those of sham group (Fig. 7a and 7d–g). LC3B and p62 immunoblotting results were further supported by their immunofluorescence assay results with both types of EPO treatments restoring their expression levels close to sham group (Fig. 7h, i). Together, these results suggest that asialo-rhuEPO^P and rhuEPO^M treatments can suppress I/R injury-induced excessive mitophagy and autophagy.

Asialo-rhuEPO^P Attenuates Apoptosis

To investigate whether asialo-rhuEPO^P-mediated attenuation of mitochondrial fission and excessive autophagy and activation of STAT5, PI3K/AKT, and/or MAPK/ERK1 signaling pathways could be translated to reduced apoptosis, several apoptotic markers were analyzed by Western blotting. As can be seen from Fig. 8a–d, asialo-rhuEPO^P, like rhuEPO^M, could significantly attenuate I/R-induced Bax/Bcl2 ratio, high levels of cyt *c*, and cleaved caspase 3 compared to I/R-saline control, indicating that asialo-rhuEPO^P and rhuEPO^M have similar suppressive effects on I/R injury-induced apoptosis. The decrease of cleaved caspase 3 levels by both EPO treatments was further supported by immunofluorescence assay results (Fig. 8e). In addition, TUNEL staining, one of major methods for detecting apoptotic programmed cell death [59], was also performed to detect apoptotic cells. TUNEL results showed that asialo-rhuEPO^P treatment (rhuEPO^M as well) reduced TUNEL positive cells by 50% compared to I/R-saline group (Fig. 8f, g). These results further reinforced anti-apoptotic effects of asialo-rhuEPO^P.

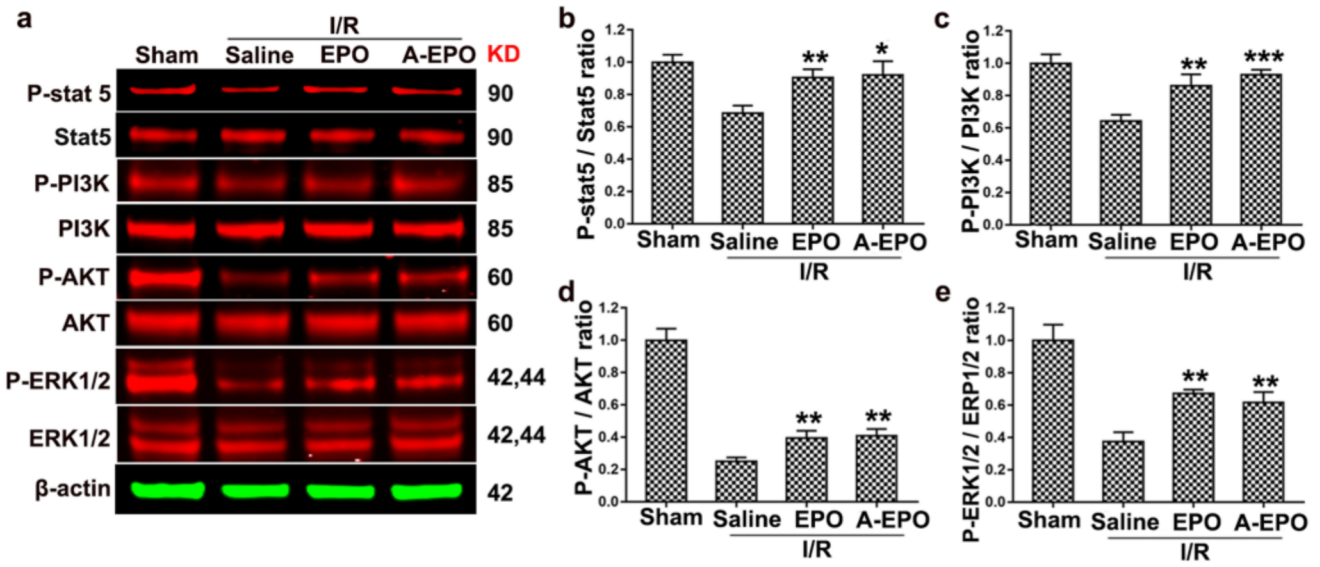

Fig. 4 The effects of asialo-rhuEPO^{P} on key proteins of three cell survival pathways. **a** Representative Western blotting results of phosphorylated and total protein content of STAT5, PI3K, AKT, and ERK1/2. β-Actin was used as an internal control. **b–e** Quantified ratios between phosphorylated (p-) and total protein of STAT5, PI3K, AKT, and ERK1/2. All data plotted are the average ± SD. *, **, ***: $p < 0.05$, 0.01, 0.001, asialo-rhuEPO^{P} (A-EPO) or rhuEPO^{M} (EPO) versus saline mice under I/R experimental conditions

Discussion

Stroke is still one of major causes of death and disability worldwide even though tremendous efforts have been made to devise new treatment strategies. Mechanical thrombectomy or treatment with thrombolytic agents remains the mainstay of treatment while the contribution from neuroprotective drug therapy is very limited [1, 4]. Many promising neuroprotectants from preclinical studies failed in ischemic stroke clinical trials. Failure of these drug candidates could be due to many reasons, but is thought to be mainly due to isolated targeting of only a single specific cellular pathway [5, 6] while the pathophysiology of stroke is complex and involves dysregulation of numerous cellular processes [2, 3]. Therefore, multimodal neuroprotectants that can target an array of key injury mechanisms in ischemic stroke are needed to provide enhanced therapy [5–7]. Wild-type EPO and its non-erythropoietic derivatives asialo-rhuEPO, CEPO, and MEPO are reported to be such kind of neuroprotectants, which exhibit excellent neuroprotective effects [11, 17, 20, 21, 23] by acting through multiple mechanisms, including antiexcitotoxic, antiapoptotic, angiogenic, and neurogenic effects [12, 13, 25, 60]. However, clinical application of erythropoietic rhuEPO^{M} for neuroprotection was discouraged because of hematopoietic activity-related adverse effects [14, 16] while limited availability of non-erythropoietic asialo-rhuEPO was a major hurdle for translating its neuroprotective potential. The data presented in this study demonstrates that asialo-rhuEPO produced using an inexpensive plant-based expression system is non-erythropoietic, non-immunogenic, and neuroprotective in animal model of cerebral I/R injury, which makes it as a promising neuroprotection candidate.

Lack of erythropoietic activity is critical for any tissue-protective EPO derivatives because any residual erythropoietic activity at high doses can increase the risk of thrombosis leading to secondary infarction and negatively affecting treatment outcome as observed in clinical trials with rhuEPO^{M} [16]. Asialo-rhuEPO^{E} is non-erythropoietic because of its short circulatory half-life resulting from removal of terminal sialic acid residues while it exhibits multiple neuroprotective functions [20, 22, 24–27]. Regarding asialo-rhuEPO^{P}, it was produced in transgenic plants naturally lacking sialylation capacity and was confirmed to lack terminal sialic acid residues [30, 43]. Its repeated injection in BALB/c mice at a dose 55 times higher than that typically used for stimulation of erythropoiesis [40] showed no increase in hemoglobin concentration and RBC levels (Fig. 1c, d). These results fully confirmed it to be non-erythropoietic. Concerns have also been raised that plant-specific sugars α (1,3)-fucose and β (1,2)-xylose residues on any plant-produced glycoproteins could elicit unwanted immunogenic responses [44–46, 61]. Our studies through immunization of BALB/c mice with asialo-rhuEPO^{P} showed no humoral responses against these unique glycan epitopes (Fig. 1f, g), indicating that it is non-immunogenic at neuroprotective and even with twofold higher doses. These results are in agreement with Chargelegue et al. [36] but differ from other studies [44, 61] where using extremely high doses (~ 100 times higher than dose used in the present study)

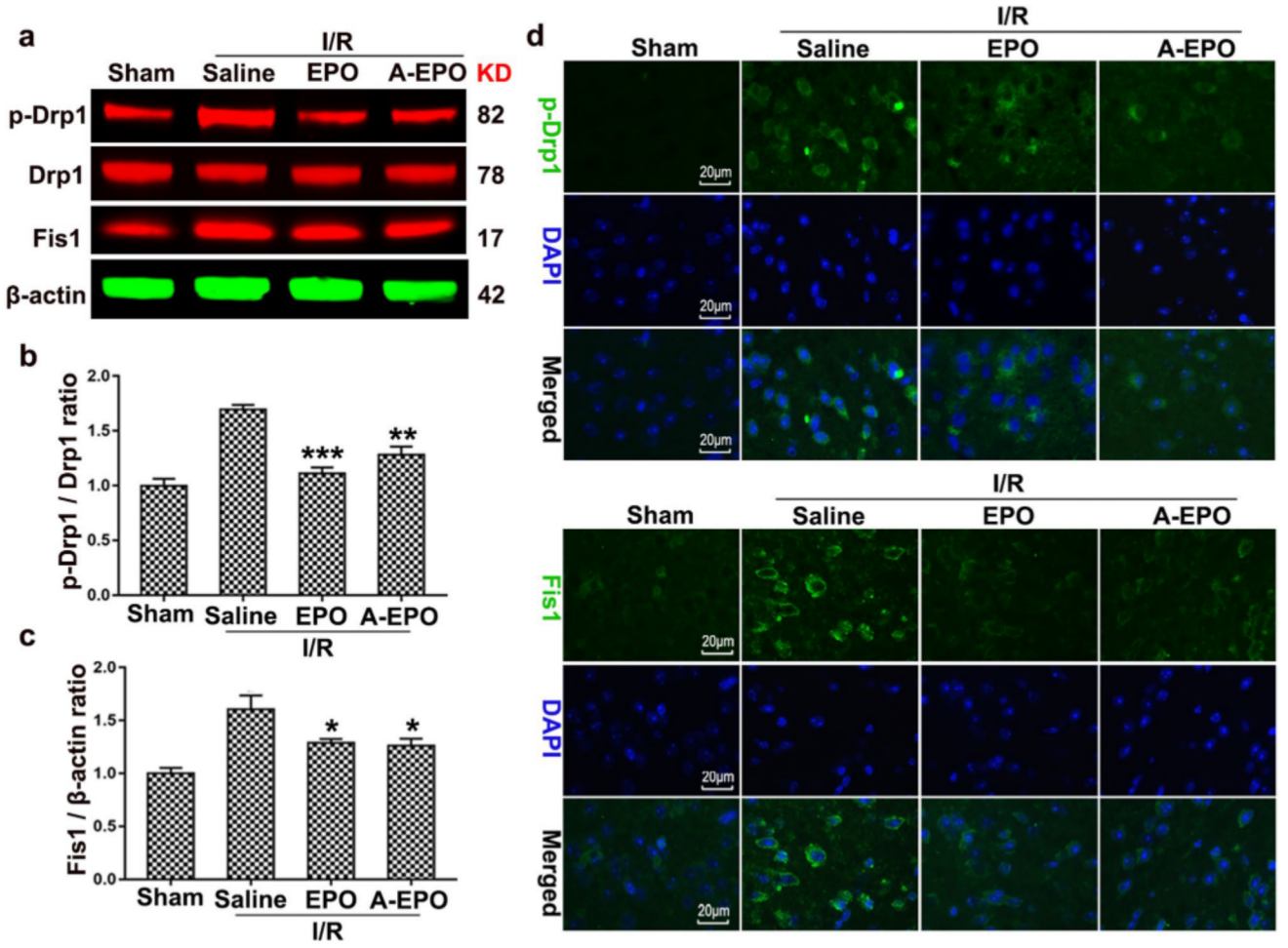

Fig. 5 The effects of asialo-rhuEPO^{P} on key proteins of mitochondrial fission. **a** Representative Western blotting results of fission related proteins Fis1 and Drp1. β-Actin was used as an internal control. **b** The ratio between p-Drp1 and Drp1. **c** The ratio between Fis1 and β-actin. All data plotted are the average ± SD. *, **, ***: $p < 0.05$, 0.01, 0.001 asialo-rhuEPO^{P} (A-EPO) or rhuEPO^{M} (EPO) versus saline mice under I/R experimental conditions. **d** Immunostaining assay results of p-Drp1 and Fis1. Brain sections were immunestained with antibody against p-Drp1 or Fis1 (green) followed by DAPI counterstaining (blue) to identify nuclei

showed induction of anti-plant glycan antibodies. Although data on induction of antibodies against these glycan epitopes in animal models remain controversial [45, 46, 61], a recent study with Taliglucerase alpha (acid β-glucosidase, an FDA approved plant-produced biopharmaceutical to treat Gaucher disease) showed that the plant-specific glycan epitopes neither induce allergic/hypersensitivity response nor affect drug efficacy [62]. Lack of erythropoietic activity and humoral response against plant-specific *N*-glycan epitopes indicates that asialo-rhuEPO^{P} is a safe drug candidate.

In this study, we further demonstrate that asialo-rhuEPO^{P} is neuroprotective with comparable efficacy to rhuEPO^{M}. This is evidenced by its ability to reduce infarct volume and edema, and improve neurological deficit scores with the same efficacy as rhuEPO^{M} in a mouse cerebral I/R injury model (Fig. 2b–e). What are then the mechanisms by which asialo-rhuEPO^{P} exerts its neuroprotective effects? Firstly, our Western blotting results showing increased phosphorylation of STAT5, PI3K/AKT, and ERK1/2 following asialo-rhuEPO^{P} treatment indicate that it restores I/R injury-impaired STAT5, PI3K/AKT, and MAPK cell survival pathways contributing to its neuroprotection as also reported previously for rhuEPO^{M} [12, 13]. STAT5 is a pro-survival factor essential for positively regulating expression of anti-apoptotic Bcl2 family members and maintaining mitochondrial membrane stability [63]. The PI3K/AKT and MAPK (via activation of ERK1/2) pathways are also well-known promoters of cell survival [64, 65], and activation of these by rhuEPO^{M} and its derivatives have been well documented to promote cell survival by maintaining mitochondrial membrane stability, and inhibiting inflammation and apoptosis [12, 13]. Thus, it appears that asialo-rhuEPO^{P} exerts its neuroprotective effects by activating STAT5, PI3K/AKT, and MAPK pathways to inhibit apoptosis.

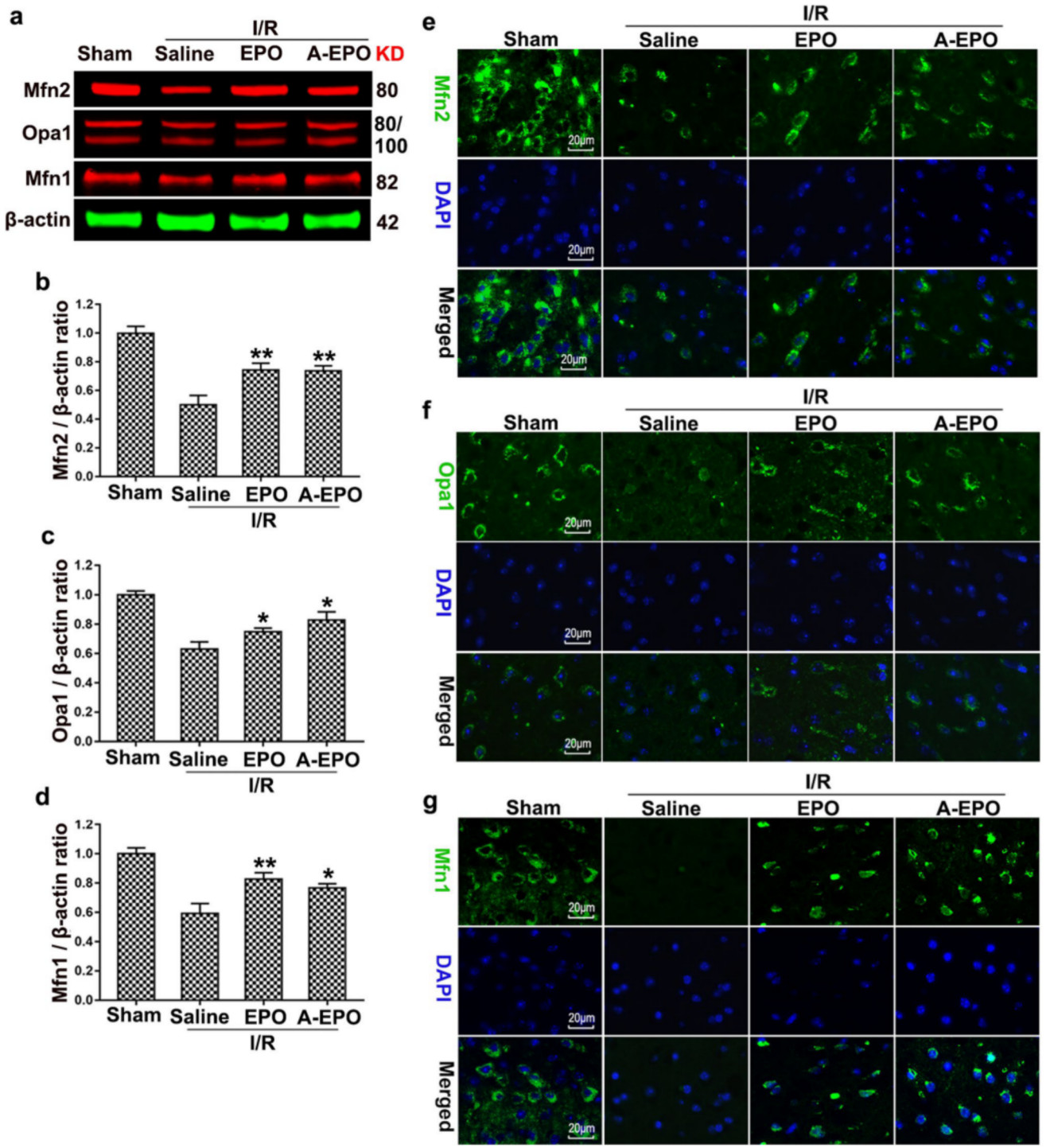

Fig. 6 The effects of asialo-rhuEPO^{P} on key proteins of mitochondrial fusion. **a** Representative Western blotting results of Mfn2, OPA1, and Mfn1. β-Actin was used as an internal control. The ratio between Mfn2 (**b**), OPA1 (**c**), or Mfn1 (**d**) and β-actin was quantified. All data plotted are the average ± SD. *, **: $p < 0.05$, 0.01 asialo-rhuEPO^{P} (A-EPO) or rhuEPO^{M} (EPO) versus saline mice under I/R experimental conditions. Immunostaining assay results of Mfn2 (**e**), OPA1 (**f**), and Mfn1 (**g**). Brain sections were immunestained with antibody against Mfn2, OPA1, or Mfn1 (green) followed by DAPI counterstaining (blue) to identify nuclei

Secondly and most importantly, our results demonstrated that asialo-rhuEPO^{P} (rhuEPO^{M} as well) exerts its neuroprotective effects by targeting mitochondrial quality control mechanisms. We discovered that asialo-rhuEPO^{P} decreased I/R injury-induced levels of mitochondrial fission-related Drp1 and Fis1 proteins (Fig. 5) and restored injury-affected

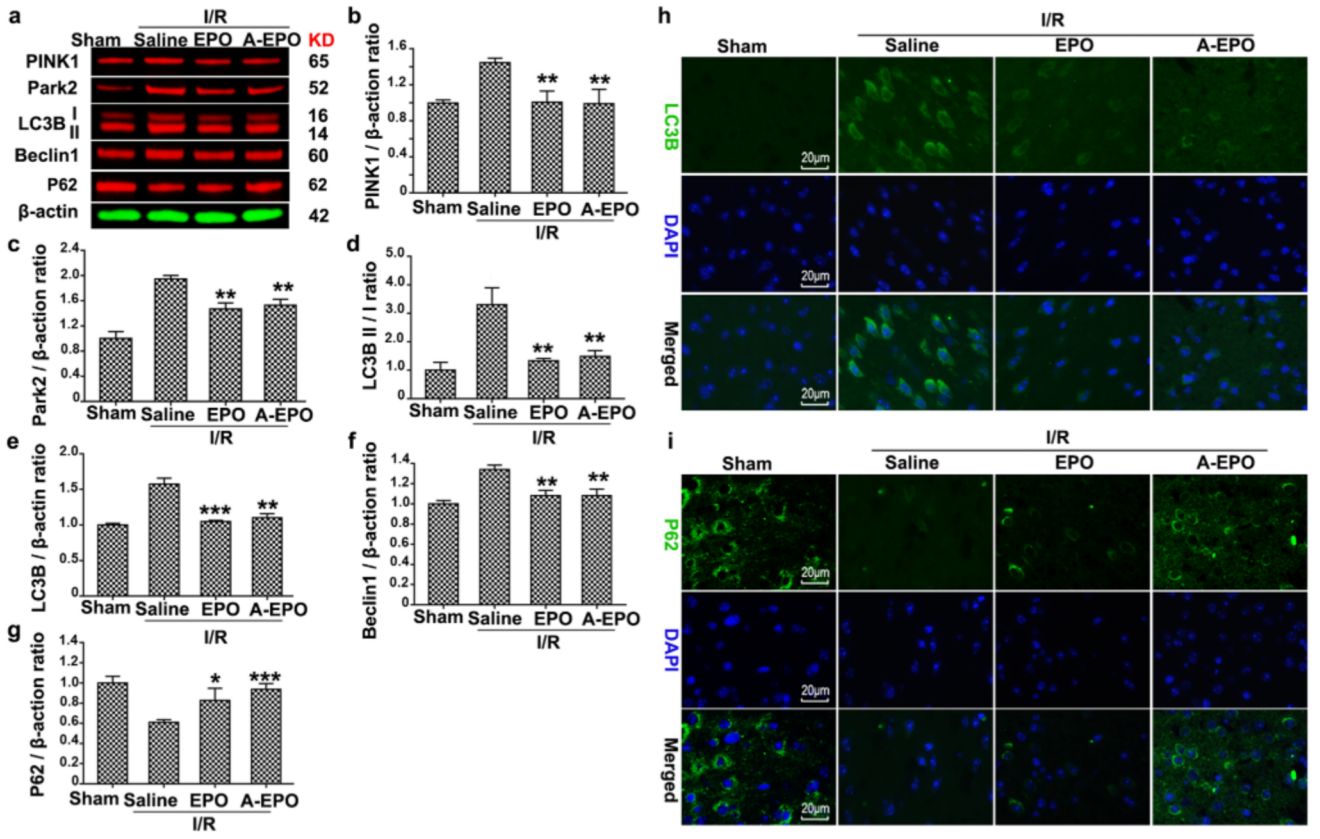

Fig. 7 The effects of asialo-rhuEPO^{P} on mitophagy and general autophagy-related markers. **a** Representative Western blotting results of PINK1, parkin, LC3B, Beclin1, and P62. β-Actin was used as an internal control. **b**, **c** The ratio between PINK1 or parkin and β-actin. **d** The ratio between LC3B II/I. **e** The ratio between LC3B and β-actin. **f**, **g** The ratio between Beclin1 or P62 and β-actin. All data plotted are the average ± SD. *, **, ***: $p < 0.05$, 0.01, 0.001 levels asialo-rhuEPO^{P} (A-EPO) or rhuEPO^{M} (EPO) versus saline mice under I/R experimental conditions. Immunostaining assay results of brain sections using antibodies against LC3B (**h**) and P62 (**i**) (green) and stained with DAPI (blue) to observe nuclei

fusion-related Mfn1, Mfn2, and Opa1 protein levels (Fig. 6). These results suggest that it may regulate mitochondrial dynamics by reducing mitochondrial fission and favoring mitochondrial fusion. This notion is further supported by our finding that asialo-rhuEPO^{P} reduced the levels of mitophagy markers PINK1 and parkin (Fig. 7a–c) since induced mitochondrial fission is followed with enhanced mitophagy to clear damaged mitochondria [53, 54]. Proper maintenance of fission–fusion balance is particularly important for neurons because neuronal mitochondria are biosynthesized in the soma and transported throughout axons and dendrites along microtubule tracks to meet end-to-end energy demands, Ca^{2+} influx, and neurotransmission [48, 49]. Excessive mitochondrial fission caused by cerebral insults can lead to mitochondrial dysfunction and induction of apoptosis by releasing pro-apoptotic factors such as cyt *c* [48, 49, 52]. Although it is not known whether asialo-rhuEPO^{P} and rhuEPO^{M} have the ability to directly modulate both mitochondrial dynamics and mitophagy, the present study at least suggests that asialo-rhuEPO^{P} may prevent cerebral ischemia-induced mitochondrial fission–fusion imbalance and autophagy alterations. In addition, some recent studies reported that activated AKT prevents osteoblast apoptosis and protects the heart from I/R injury by modulating mitochondrial dynamics [66, 67]. Since we also observed activation of PI3K and AKT by both asialo-rhuEPO^{P} and rhuEPO^{M} treatments (Fig. 4a, c, d), we speculate that these EPO-mediated activations might be involved in the modulation of I/R injury impaired mitochondrial dynamics. However, further work is needed to understand how PI3K and AKT regulate impaired mitochondrial dynamics in neurons. Irrespective of the molecular mechanism(s) involved, our finding that asialo-rhuEPO^{P} can restore I/R-disturbed mitochondrial fission–fusion proteins is significant because it could also be used to target mitochondrial dysfunction resulting from impaired mitochondrial dynamics in neurodegenerative diseases [34, 66].

Finally, we confirmed that asialo-rhuEPO^{P} and rhuEPO^{M} suppress excessive autophagy and inhibit apoptosis. Both Western blotting and immunofluorescence results showed

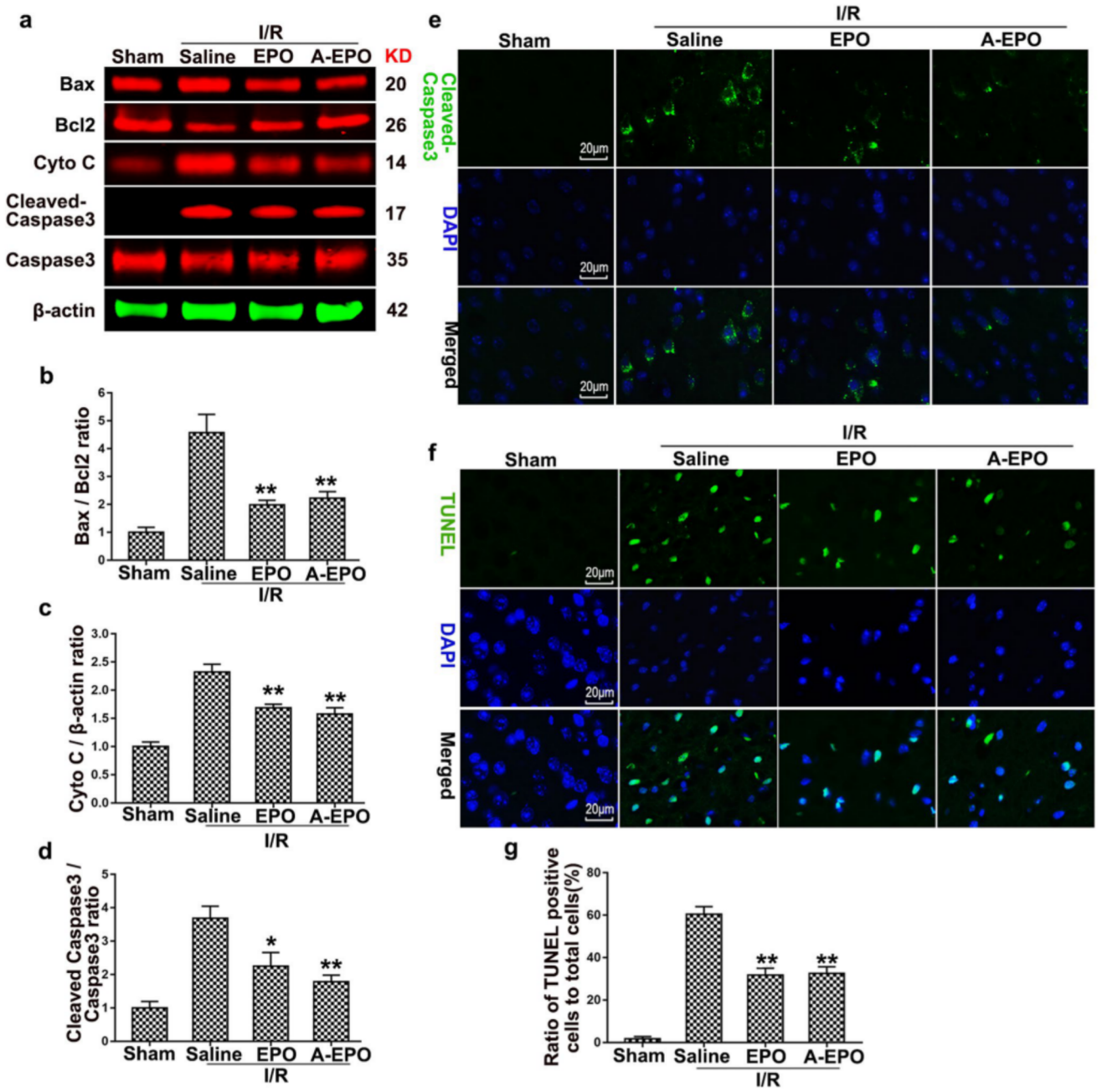

Fig. 8 The effects of asialo-rhuEPO^{P} on apoptosis. **a** Representative Western blotting results of apoptosis-related proteins Bax, Bcl2, Cyt *c*, and caspase 3. β-Actin was used as an internal control. **b** The ratio between Bax and Bcl2. **c** The ratio between cyt *c* and β-actin. **d** The ratio between cleaved and total caspase 3. **e** Immunostaining assay results of brain sections using an antibody against cleaved caspase 3 (green) and stained with DAPI (blue) to observe nuclei. **f** Representative TUNEL staining results of brain sections from different treatments. **g** Ratio of TUNEL-positive cells. Data were plotted as the average ± SD. *, **: $p < 0.05$, 0.01 levels asialo-rhuEPO^{P} (A-EPO) or rhuEPO^{M} (EPO) versus saline mice under I/R experimental conditions

that asialo-rhuEPO^{P} decreased excessive autophagy induced by I/R injury (Fig. 7d–i). Since excessive autophagy has been shown to contribute to neuron death in cerebral ischemia [68], its reduction by asialo-rhuEPO^{P} could benefit neuronal cell survival. Moreover, given the role of STAT5, AKT, and ERK1/2 in inhibiting apoptosis by targeting pro-survival (Bcl2, MCL1) and pro-death (BAD, BIM, BMF, PUMA, and BIK) proteins on mitochondria [63, 69], it is highly likely that asialo-rhuEPO^{P} mediates its neuroprotective effects through activation of STAT5, PI3K/AKT, and MAPK pathways leading to stabilization of mitochondria and inhibition

of apoptosis. Its anti-apoptotic effects were further verified by our Western blotting and TUNEL assay results (Fig. 8).

As for the observed similar neuroprotective effects and mechanisms observed within 24 h between asialo-rhuEPO^{P} and rhuEPO^{M}, it is not surprising given the fact that there are only minor differences between them in their *N*-glycan chains, with the former lacking terminal sialic acid residues. Asialo-rhuEPO, unlike other EPO derivatives (CEPO and EPO peptides) which lack hematopoietic receptor (EPOR)2-binding sites [17, 18, 21], retains these binding sites [70] and can bind with even higher affinity than wild-type EPO [71]. Therefore, it is possible that asialo-rhuEPO^{P} mediates its antiapoptotic effects through (EPOR)2. In addition, an alternative receptor consisting of EPOR chain and beta common receptor chain (EPOR-βcR) [72] and even some other tissue-protective receptors [73] have also been proposed to specifically mediate tissue protective effects of EPO and its non-erythropoietic derivatives. Asialo-rhuEPO^{P} has the same polypeptide sequence as rhuEPO^{M} and can bind to these proposed tissue-protective specific receptors if they are real. However, the roles of these receptors in mediating tissue-protective effects of EPO and its derivatives are still controversial [74]. It remains to be determined which receptor(s) EPO and asialo-rhuEPO^{P} utilize to mediate their neuroprotective effects. Besides, it is also possible to find some asialo-rhuEPO^{P} unique protective mechanisms by performing genome-wide studies and the investigation of long-term effects since asialo-rhuEPO^{P} and rhuEPO^{M} have different terminal sugar residues with opposite protein charges and the former lacks erythropoietic activity.

In summary, we observed no erythropoietic activity with asialo-rhuEPO^{P} or no humoral response to its plant-specific sugars indicating it to be safe for clinical investigation. Intravenous administration of asialo-rhuEPO^{P} at the initiation of reperfusion protected mouse brains from I/R injury by improving neuron survival. Furthermore, its neuroprotective mechanisms involve activation of three cell survival pathways leading to stabilization of mitochondrial fission–fusion balance, prevention of excessive mitophagy and autophagy, and inhibition of apoptosis. Therefore, this study suggests that asialo-rhuEPO^{P} as a non-erythropoietic and non-immunogenic, and neuroprotective agent holds great clinical translational potential for stroke management.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12975-021-00943-z.

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Author Contribution F.S.K, D.C.S, P.A.L, and J.X. designed research; M.H, F.S.K, C.-Y.H, and J.Z. performed the experiments; M.H, F.S.K, P.A.L., J.Z. L.J., D.C.S, and J.X. analyzed the data. M.H, F.S.K., and J.X. drafted the manuscript with input from all authors.

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Data Availability All data associated with this study are present in the paper, which have been confirmed by authors to comply with field standards.

Code Availability Software application and custom code used to support the published claims have been confirmed by authors to comply with field standards.

Declarations

Ethics Approval This study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Association and Accreditation of Laboratory Animal Care International guidelines and under the authorization of the Institutional Animal Care and Use Committee at the North Carolina Central University, Durham, NC, USA.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest JX, FK, and C-Y H are inventors of filed patent "Methods for the production of cytoprotective asialo-erythropoietin in plants and its purification from plant tissues" (PCT NUMBER: US2013031382, pending).

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