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Electroacupuncture ameliorates neuronal injury by Pink1/Parkin-mediated mitophagy clearance in cerebral ischemia-reperfusion

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ABSTRACT

The accumulation of dysfunctional mitochondria induced by the impairment of the autophagy-lysosome pathway (ALP), especially mitophagy is an important cause of cerebral ischemia-reperfusion (I/R) injury. Electroacupuncture (EA) exerts remarkable effects in treating ischemic stroke; however, the detailed mechanism remains unclear. In this study, rats were treated with mitochondrial permeability transition pore (mPTP) opening inhibitor, peroxynitrite ($ONOO^-$) scavenger, or selective inhibitor of mitophagy activation during 2-h middle cerebral artery occlusion (MCAO) followed by 24 h of reperfusion in combination with EA treatment. RNA-Seq analysis showed that EA treatment in cerebral I/R was linked to the autophagosome, the PI3K/Akt signaling pathway and metabolic pathways. We found that I/R resulted in significantly mitochondrial function impairments including decreased mitochondrial membrane potential (MMP) and ATP levels, aggregation of damaged mitochondria, excessive nitro/oxidative stress, PI3K/Akt/mTOR-mediated ALP dysfunction and deficiency of Pink1/Parkin-mediated mitophagy clearance. The treatment with EA, cyclosporine-A (CsA, a potent inhibitor of mPTP opening) or FeTMPyP (a type of $ONOO^-$ scavenger) could significantly increase MMP and/or ATP levels, improve mitochondrial function and decrease neuronal injury. At the same time, EA also improved ALP dysfunction and the deficiency of mitophagy clearance; however, mitochondrial division inhibitor-1 (Mdivi-1, a selective inhibitor of mitophagy activation) blocked mitophagy clearance and aggravated neuronal injury. Taken together, EA ameliorates nitro/oxidative stress-induced mitochondrial functional damage and decreases the accumulation of damaged mitochondria via Pink1/Parkin-mediated mitophagy clearance to protect cells against neuronal injury in cerebral I/R.

1. Introduction

Ischemic stroke is one of the most common causes of death and disability worldwide. The reconstruction of blood flow is the most effective method to treat cerebral ischemia. However, in some cases, sudden reperfusion can be accompanied by a number of complications, including hemorrhagic transformation, blood-brain barrier destruction, and a large amount of edema, which may cause further injury, known as cerebral ischemia-reperfusion (I/R) injury [1]. Studies have confirmed that oxidative stress is an important pathogenesis of ischemic stroke, and excessive reactive oxygen species (ROS) produced by mitochondria are considered to be the main cause of oxidative stress [2].

Mitochondria are organelles that act as the oxidative energy centers and are necessary for cell survival, whereas aging or damaged mitochondria are the sources of toxic ROS [3]. Furthermore, ROS can aggravate mitochondrial injury and lead to the accumulation of damaged mitochondria, resulting in neurodegenerative diseases. Thus, cells must clear damaged mitochondria to stop ROS accumulation. Autophagy is a key self-repair mechanism that maintains cellular homeostasis by degrading stress-related damaged organelles and misfolded proteins [4]. The cells maintain their mitochondrial quality mainly through mitophagy, which is mediated by the Pink1/Parkin pathway [5]. After MMP loss or the accumulation of damaged mitochondria, phosphatase and tensin homolog (PTEN)-induced putative kinase protein 1 (Pink1) localizes to the mitochondrial outer membrane, where it promotes the recruitment of the E3 ubiquitin ligase Parkin to the mitochondria [6]. Parkin promotes mitochondrial membrane protein ubiquitination. Then, mitochondria are wrapped by autophagosomes via an interaction between the autophagy receptor p62/SQSTM1 (Sequestosome 1) and LC3 (microtubule-associated protein 1 light chain 3). Autophagosomes eventually fuse with lysosomes to form autolysosomes, which target mitochondria for autophagy clearance. Previous studies have shown that in neurons, basal autophagy can prevent the accumulation of damaged organelles and misfolded

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proteins, thereby protecting neuronal function and preventing the occurrence of neurodegenerative diseases [7,8]. In addition, autophagy is a major protective mechanism that supports cell survival following exposure to multiple stress responses [9]. Moreover, the protective effects of autophagy during cerebral I/R may be attributable to mitophagy-related mitochondrial clearance [10]. Therefore, the clearance of damaged mitochondria allowed by increased autophagy and mitophagy levels has substantial potential for the treatment of ischemic stroke.

Electroacupuncture (EA) is a technique that combines traditional acupuncture with electrical stimulation. Due to the simplicity of the operation, its high level of safety and its few side effects, EA has been used for many neurological diseases, including stroke [11]. Although its effects are obvious, its mechanisms are not clear. Previous study indicated that a pretreatment with EA induced rapid tolerance to subsequent focal cerebral ischemia [12]. Acupuncture can also effectively restore lysosome levels and induce autophagy clearance, resulting in the recovery of dopamine (DA) neurons and the improvement of motor function [13]; however, the specific autophagy pathways and key regulators that are induced by EA remain unclear.

In this study, we focus on the effects of EA treatment on mitochondria, and found that EA could enhance Pink1/Parkin-mediated mitophagy clearance, decrease the accumulation of damaged mitochondria, and ameliorate nitro/oxidative stress-induced mitochondrial functional damage in cerebral I/R. Our findings provide a better understanding of the molecular mechanism of EA as a treatment for ischemic stroke.

2. Materials and methods

2.1. Animals

Adult male Sprague Dawley (SD) rats weighing 220–250 g were supplied by Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). This study was performed under the supervision of the Animal Care and Use Committee of Peking Union Medical College Hospital.

2.2. Experimental design

2.2.1. Experiment I

To verify the roles of EA in cerebral I/R injury in addition to the specific signaling pathways involved in EA, SD rats were randomly divided into three groups: sham, I/R and EA. Staining with 2,3,5-triphenyltetrazolium chloride (TTC), Garcia JH scoring and Nissl staining were used to evaluate the cerebral ischemic area, neurological function and neuronal damage, respectively. RNA sequencing (RNA-Seq) was used to analyze the specific regulatory mechanisms of EA.

2.2.2. Experiment II

To study the effects of EA on damage to mitochondrial function and the accumulation of damaged mitochondria during cerebral I/R, SD rats were randomly divided into four groups: sham, I/R, EA and CsA. MMP and ATP levels were detected to explore mitochondrial function. CsA (a potent inhibitor of mPTP opening) was used to verify the important roles of mitochondria in the process of cerebral I/R. In addition, mitochondrial markers were detected to quantify the accumulation of damaged mitochondria.

2.2.3. Experiment III

To study the effects of EA on nitro/oxidative stress as well as the effects of nitro/oxidative stress on mitochondrial function during cerebral I/R, SD rats were randomly divided into four groups: sham, I/R, EA and FeTMPyP groups. Several key enzymes and products were detected to determine the status of nitro/oxidative stress. FeTMPyP (a type of $ONOO^-$ scavenger) was used to study the effects of nitro/oxidative stress on mitochondrial function.

2.2.4. Experiment IV

To study the effects of EA on PI3K/Akt/mTOR-mediated ALP dysfunction and deficiency in Pink1/Parkin-mediated mitophagy clearance during cerebral I/R, SD rats were randomly divided into five groups: sham, I/R, EA, mitochondrial division inhibitor-1 (Mdivi-1) and Mdivi-1 + EA. Key regulatory proteins and mitophagy-specific proteins were evaluated at various stages of ALP to determine the status of autophagy/mitophagy. Mdivi-1 (a selective inhibitor of mitophagy activation) was used to verify the important roles of mitophagy in neuronal injury in the process of cerebral I/R.

2.3. Other methods

A detailed Methods section is available at supplementary material for all of these procedures, including cerebral I/R model, EA and drug treatment, cerebral infarct size measurement, Nissl staining, RNA sequencing, mitochondrial isolation, detection of MMP and ATP Levels, Western blot analysis, RNA extraction and real-time PCR, immunofluorescence, measurement of various nitro/oxidative stress indexes.

2.4. Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed with SPSS13.0. Comparisons between groups were made using one-way ANOVA followed by Bonferroni post hoc test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. EA attenuated cerebral I/R injury in a model of MCAO

To study the effects of EA on cerebral I/R injury, we adopted a model of 2-h MCAO followed by 24 h of reperfusion in combination with EA treatment (Fig. 1a). We found that in the I/R group, infarct size was significantly higher and that EA treatment partially reduced infarct size after cerebral I/R ($P < 0.05$, Fig. 1b). Garcia JH scoring indicated that compared with the sham group, in the I/R group, the scores were significantly lower ($P < 0.001$), while EA treatment partly increased the scores ($P < 0.01$, Fig. 1c). Moreover, in the sham group, Nissl staining revealed that in the cortex and the cornu ammonis 1(CA1) region of the hippocampus, the cells were arranged in an orderly and regular manner, and neuronal structure was complete, including a regular nucleus and abundant Nissl bodies in the cytoplasm. However, in the I/R group, neurons were arranged in a disordered fashion, and some neurons were shrunken and deformed. Nucleoconstriction, dissolution, and cell swelling were observed, and there were fewer or no Nissl bodies (Fig. 1e and f). EA treatment significantly improved neuronal damage and increased the number of $Nissl^+$ neurons ($P < 0.05$, Fig. 1d). These results suggested that EA attenuated cerebral I/R injury in a model of MCAO.

3.2. EA played comprehensive regulatory roles in cerebral I/R

To identify the mechanisms by which EA treatment affects cerebral I/R injury, we conducted an RNA sequencing analysis. The results showed that compared with the sham group, 320 genes were upregulated in the I/R group, and 63 genes were downregulated. Compared with the I/R group, 250 genes were upregulated in the EA group, and 279 genes were downregulated (Fig. 2a). Moreover, these differences were significant (FDR < 0.05, $|\log FC| > 1$, Fig. 2b). Next, we analyzed these differentially expressed genes according to the biological processes, cellular components and molecular functions affected. The results show that compared with the sham group, in the I/R group, the number of upregulated genes was significantly higher, whereas the number of downregulated genes was lower, indicating a clear imbalance (Fig. 2c). After EA treatment, the number of downregulated

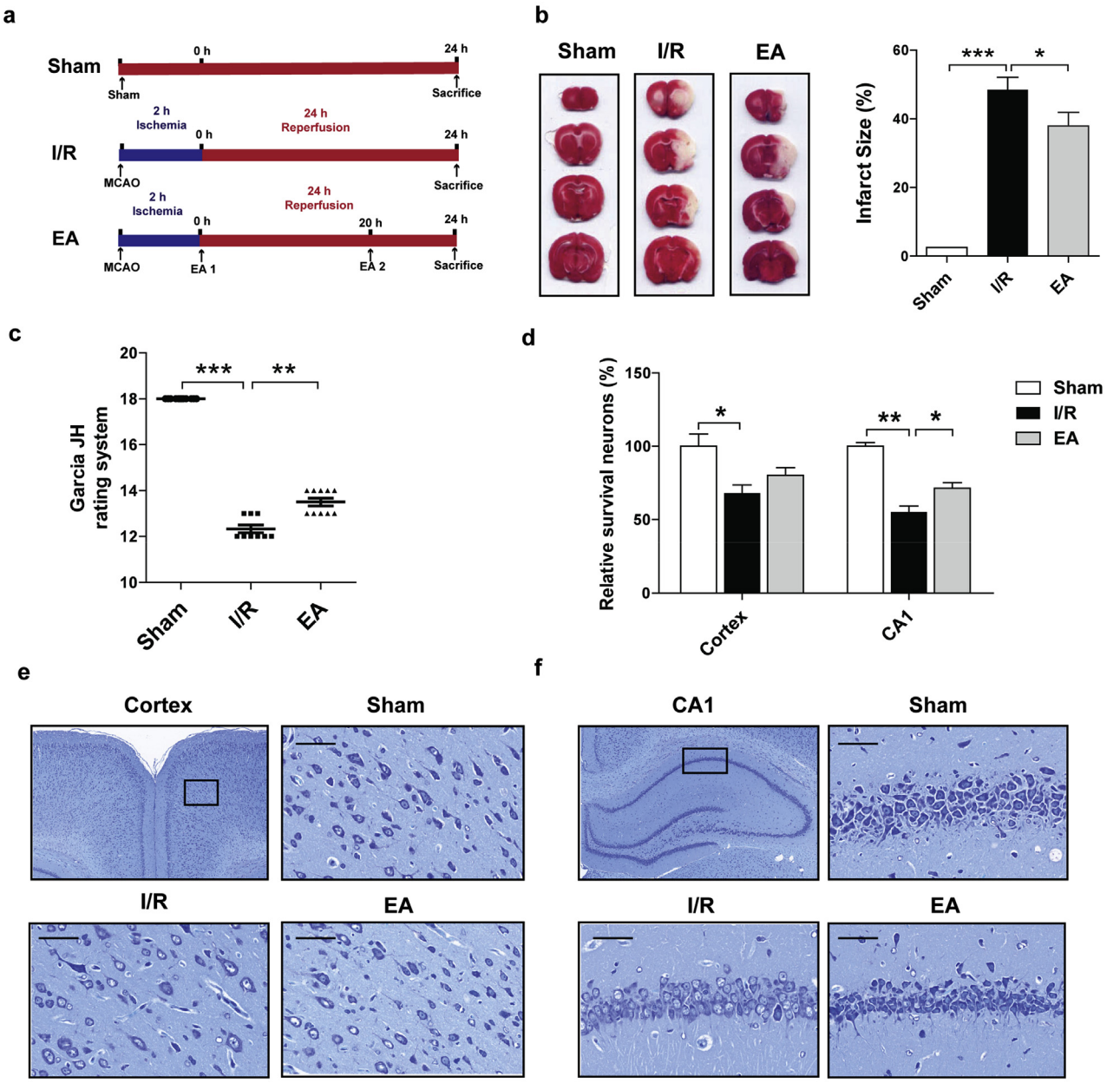

Fig. 1. EA attenuated cerebral I/R injury in an MCAO model. (a) Diagrammatic drawing of experimental procedures and corresponding EA treatment. (b) Cerebral infarct size assessed by TTC staining, n = 5 each. (c) Neurological function assessed by Garcia JH scoring, n = 10. (d) The relative number of positive neurons by Nissl staining in the cortex (e) and CA1 region of the hippocampus (f), n = 3 each, scale bar = 50 μm. Data are expressed as the mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

genes dramatically increased and reached a relatively balanced state across all three of the evaluated aspects (Fig. 2d). These results provide further evidence showing that EA plays comprehensive regulatory roles in cerebral I/R.

3.3. EA treatment may affect mitochondria and autophagy/mitophagy in cerebral I/R

To identify the specific regulatory pathways affected by EA treatment, the genes with significant differences were analyzed in a trend analysis. These genes were divided into eight categories (profile 0–7, Fig. 3a), and the results show that only four trends were significant; these included profile 3, profile 4, profile 6 and profile 7 ($P < 0.05$, Fig. 3b). By calculating the percentage of each profile gene in the corresponding pathways, we found that the pathway commonly affected by the above four gene trends was signal transduction (Fig. 3c). Then, by pathway enrichment, we identified three significant pathways for EA treatment; these included phagosomes (a possible membrane source for early autophagosomal precursor structures), the PI3K/Akt signaling pathway (key autophagy regulators) and metabolic pathways (Fig. 3d), which may involve mitochondrial function (the oxidative energy metabolism center of the cell). Therefore, we speculated that EA treatment might be related to mitochondria and autophagy/mitophagy in cerebral I/R.

3.4. EA improved mitochondrial injury and damaged mitochondrial accumulation in cerebral I/R

Based on the above hypothesis, we next studied mitochondrial function. The mPTP is necessary to maintain mitochondrial function

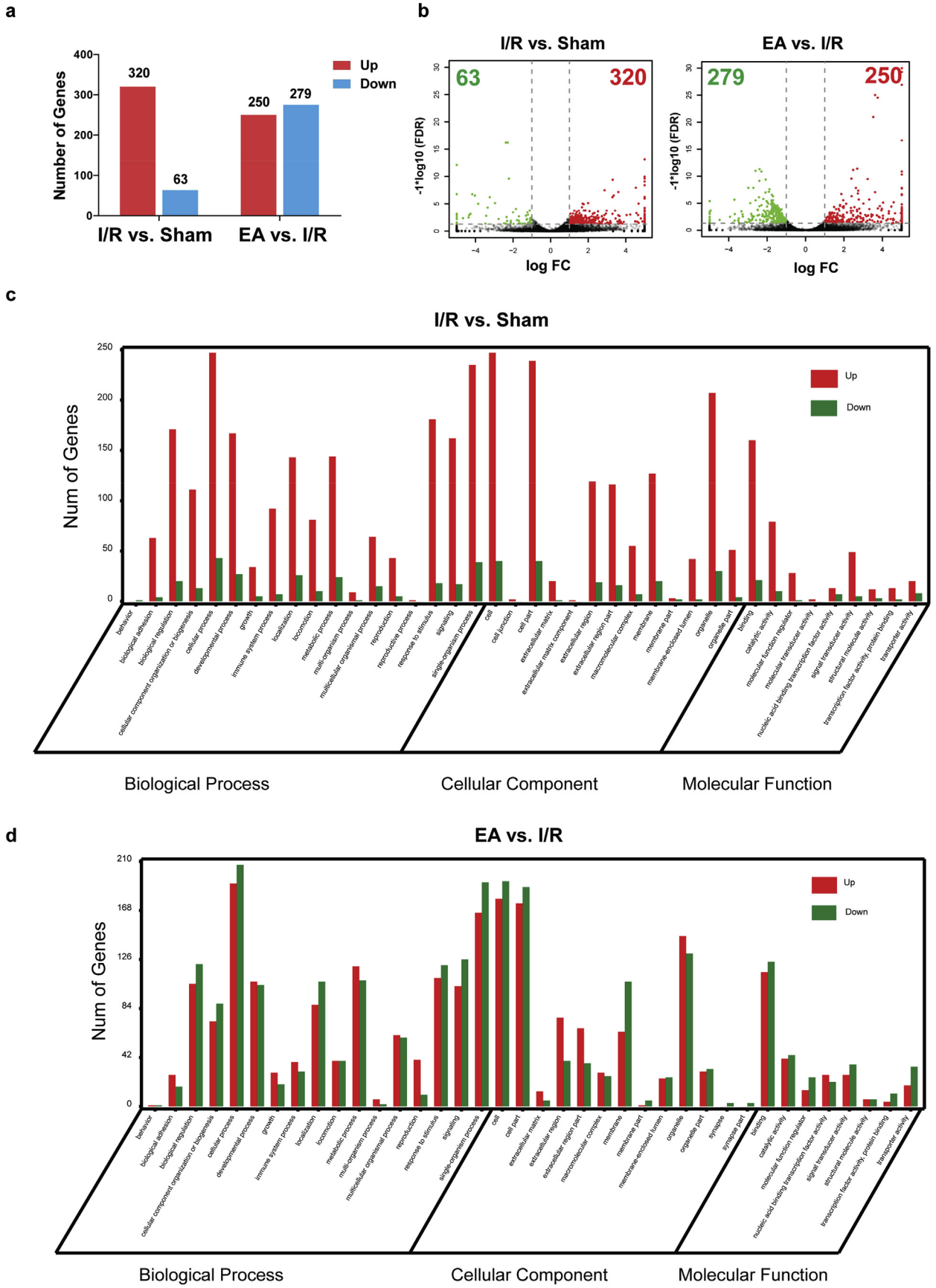

Fig. 2. EA played comprehensive regulatory roles in cerebral I/R. (a) The number of upregulated and downregulated genes in three experimental groups. (b) Volcano plot of genes with significant differences, FDR < 0.05, |log FC| > 1. (c and d) Gene Ontology (GO) function analysis of genes with significant differences in three experimental groups. Three independent samples were tested in each experimental group.

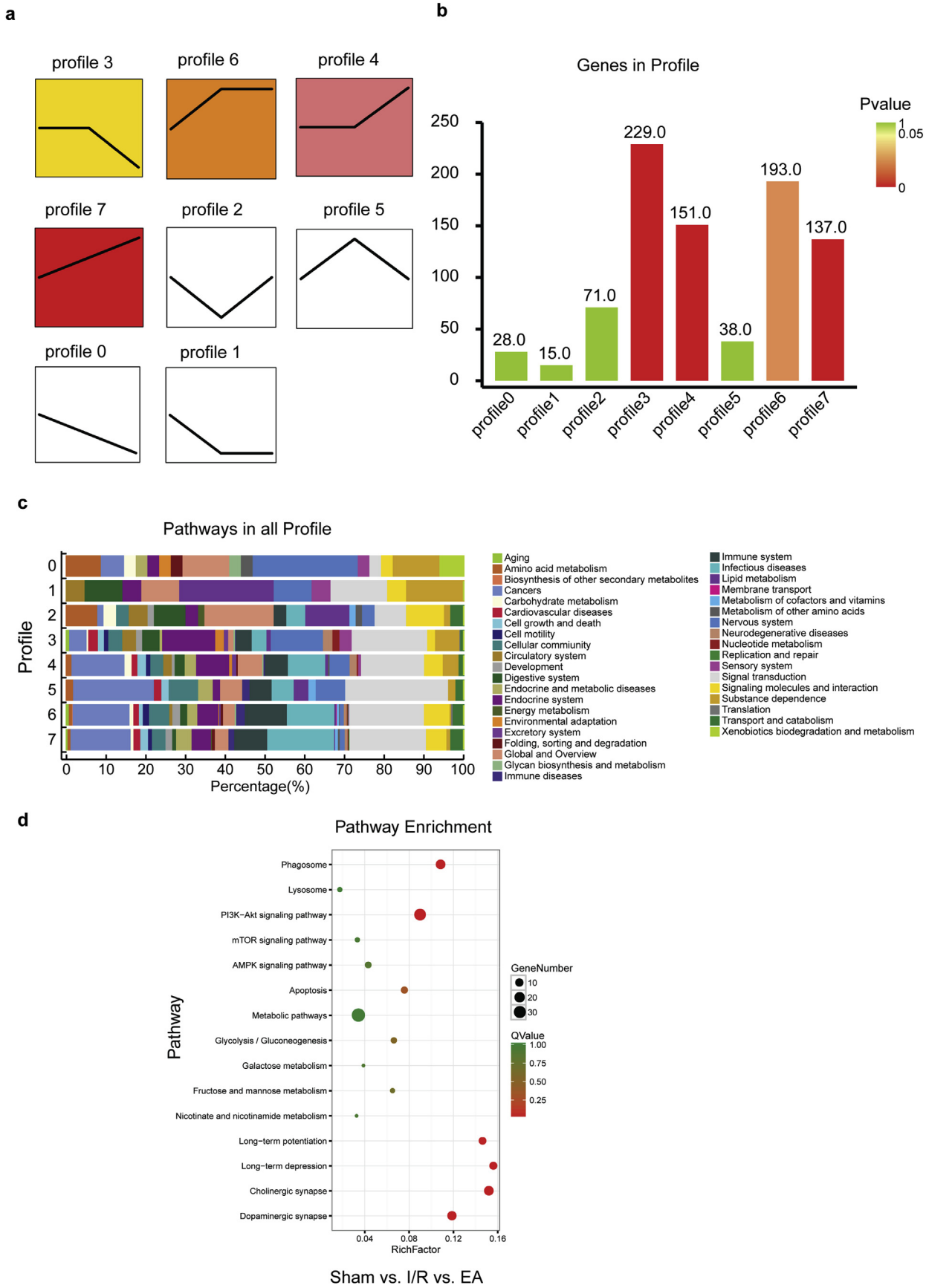

Fig. 3. EA treatment may be related to mitochondria and autophagy/mitophagy in cerebral I/R. (a) All trends (profile 0–7) of genes with significant differences. (b) Histogram of the four trends (red) with significant differences, $P < 0.05$. (c) Pathway-related gene distribution (%) in all profiles. (d) Bubble diagram of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. Three independent samples were tested in each experimental group.

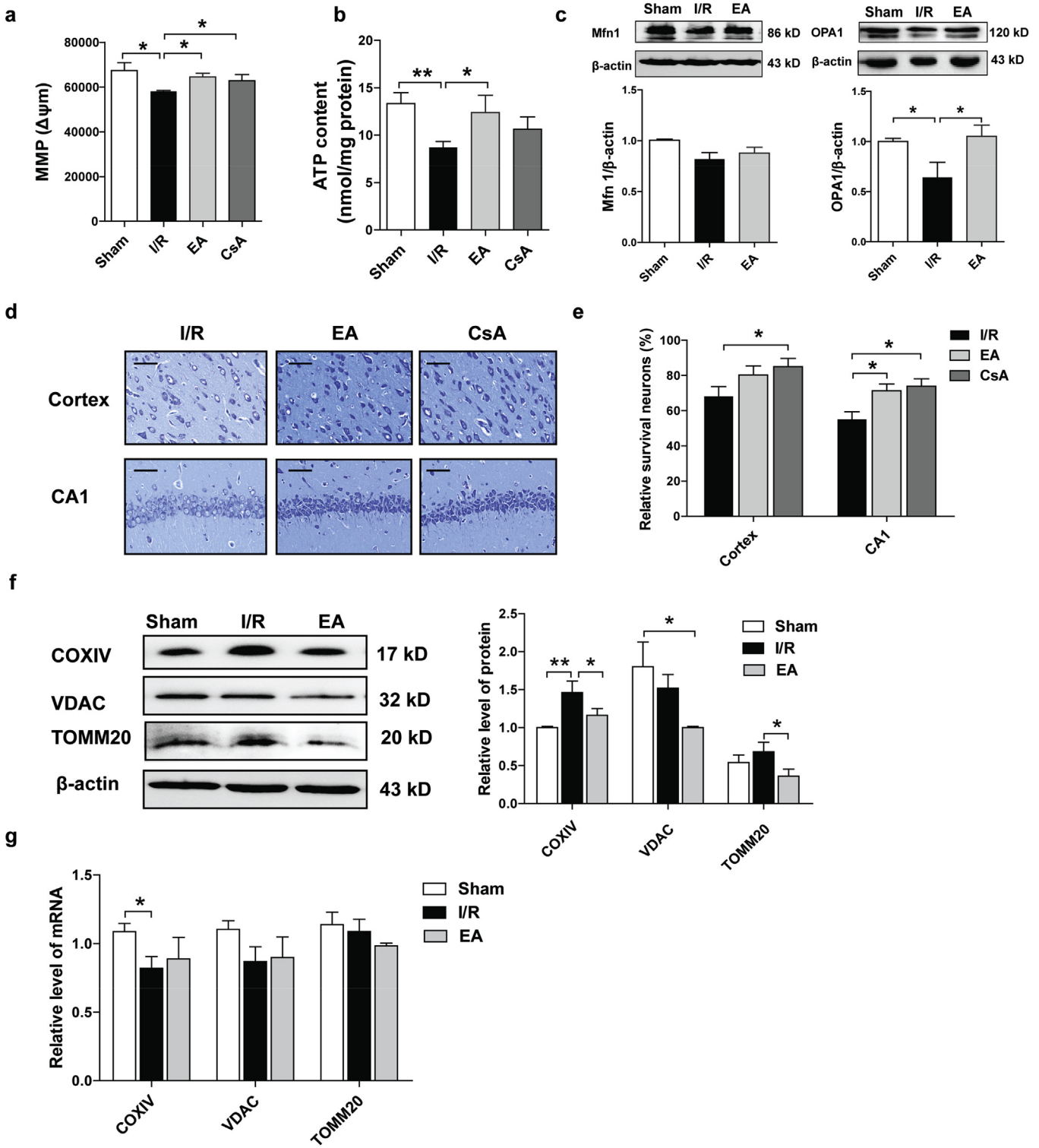

Fig. 4. EA improved mitochondrial injury and damaged mitochondrial accumulation in cerebral I/R. (a) Mitochondrial membrane potential (MMP) assessed using a JC-1 fluorescence probe, n = 5 each. (b) The level of ATP in each experimental group, n = 5 each. (c) The expression of two proteins related to mitochondrial fusion (Mfn1 and OPA1) by Western blot, n = 5 each. (d and e) The relative number of positive neurons by Nissl staining in the cortex and CA1 region of the hippocampus, n = 3 each, scale bar = 50 μm. (f) The expression of mitochondrial markers (COX IV, VDAC and TOMM20) by Western blot, n = 5 each. (g) The mRNA levels of *COX IV*, *VDAC* and *TOMM20* by real-time PCR, n = 5 each. Data are expressed as the mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$.

[14]. Its opening can lead to a reduction in MMP. In this study, MMP was significantly lower in the I/R group than in the sham group but significantly increased after EA and CsA (a potent inhibitor of mPTP) treatment ($P < 0.05$, Fig. 4a). Meanwhile, EA treatment also reversed the decrease in ATP content observed in the I/R group ($P < 0.05$, Fig. 4b). Mitochondria are constantly undergoing fusion and fission to maintain a normal structure and functions [15]. Therefore, we examined the expression of two proteins related to mitochondrial fusion: mitofusin 1 (Mfn1) and optic atrophy 1 gene protein (OPA1). Although Mfn1 only showed a downward trend in the I/R group, the expression

of OPA1 was significantly decreased in the I/R group and was reversed after EA treatment ($P < 0.05$, Fig. 4c). We also found that the number of Nissl$^+$ neurons in the cortex and CA1 region of the hippocampus was significantly higher in the CsA treatment group than in the I/R group ($P < 0.05$, Fig. 4d and e), suggesting that maintaining the stability of the mitochondrial membrane could reverse neuronal injury.

The clearance of damaged mitochondria is essential for mitochondrial homeostasis in neurons. Thus, we examined the expression of the mitochondrial markers: cytochrome *c* oxidase IV (COX IV), voltage-dependent anion channel (VDAC) and translocase of outer mitochondrial membrane 20 homolog (TOMM20). The results showed that compared to the sham group, in the I/R group, COX IV expression was significantly higher ($P < 0.01$, Fig. 4f), VDAC expression was not significantly changed, and TOMM20 expression showed an obvious upward trend. Additionally, EA treatment significantly decreased the levels of COX IV and TOMM20 ($P < 0.01$, Fig. 4f). However, this loss of mitochondrial mass could also be caused by mitochondrial clearance or by a decrease in mitochondrial biogenesis. To eliminate biogenesis reduction as a contributing factor, we determined the mRNA levels of *COX IV*, *VDAC* and *TOMM20*. In contrast to the sham group, in the I/R group, the mRNA level of *COX IV* was lower ($P < 0.05$), and none of the mRNA levels of these mitochondrial markers were significantly downregulated by EA treatment (Fig. 4g). Overall, the above results showed that EA abrogated mitochondrial injury and the accumulation of damaged mitochondria in cerebral I/R.

3.5. EA ameliorated nitro/oxidative stress-induced mitochondrial injury in cerebral I/R

Lipid peroxidation causes the most serious damage to the mitochondrial membrane; we therefore tested the effects of EA on nitro/oxidative stress in these experiments. Our results showed that in the I/R group, the levels of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) (the main source of superoxide ($O_2^{\cdot-}$)), ROS and malondialdehyde (MDA) were significantly higher (Fig. 5a–c), while the activity of the antioxidant enzyme superoxide dismutase (SOD) was significantly lower ($P < 0.01$, Fig. 5d). Additionally, EA treatment significantly reduced NOX, ROS and MDA levels and increased SOD activity (Fig. 5a–d). In addition, we also found that the level of inducible nitric oxide (NO) synthase (iNOS) (the main source of NO in cerebral I/R) was significantly higher in the I/R group ($P < 0.05$, Fig. 5e) and that EA treatment partly decreased this increase in iNOS levels. Moreover, the level of 3-nitrotyrosine (3-NT) (a marker of $ONOO^-$, the reaction products of NO and $O_2^{\cdot-}$) was significantly higher in the I/R group ($P < 0.01$), and the level of 3-NT was significantly decreased by EA treatment ($P < 0.05$, Fig. 5f). Additionally, FeTMPyP (a type of $ONOO^-$ scavenger) significantly increased the MMP level after cerebral I/R ($P < 0.05$, Fig. 5g), although there was no significant change in ATP content (Fig. 5h), suggesting that the inhibition of nitro/oxidative stress ameliorated mitochondrial injury in cerebral I/R. The results presented here indicate that EA ameliorates nitro/oxidative stress-induced mitochondrial injury in cerebral I/R.

3.6. EA improved PI3K/Akt/mTOR-mediated ALP dysfunction in cerebral I/R

The ALP degrades damaged organelles and misfolded proteins, and the results described above also suggest that EA treatment may be related to the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway (key autophagy regulators). Therefore, we studied the effects of EA treatment on PI3K/Akt/mammalian target of rapamycin complex (mTOR)-mediated ALP in cerebral I/R. We found that the expression levels of PI3K and Akt showed a downward trend, while the expression of mTOR decreased significantly in the I/R group ($P < 0.05$). After EA treatment, mTOR expression was further decreased ($P < 0.01$, Fig. 6a). In addition, compared with the sham group, in the I/R group, the protein levels of LC3, Rab7 and p62 were significantly higher, and lysosome-associated membrane protein type 2 (LAMP-2) protein level was significantly lower ($P < 0.05$). Additionally, EA treatment significantly reduced Rab7 and p62 levels ($P < 0.05$) and partially increased the level of LAMP-2 (Fig. 6b). Immunofluorescence results also showed that EA increased the expression of LC3 in the CA1 region of the hippocampus after cerebral I/R ($P < 0.05$, Fig. 6c). The above results demonstrate that cerebral I/R inhibits the PI3K/Akt/mTOR signaling pathway and enhances the formation of autophagosomes; however, there was insufficient autophagosome degradation. EA rescued PI3K/Akt/mTOR-mediated ALP dysfunction in cerebral I/R.

3.7. EA increased Pink1/Parkin-mediated mitochondrial clearance in cerebral I/R

The Pink1/Parkin-mediated mitophagy is a specific pathway that degrades damaged mitochondria. Therefore, we detected the protein expression levels of components on this pathway. We found that compared with the Sham group, in the I/R group, the protein levels of Drp1 and Parkin were significantly lower ($P < 0.01$), while Pink1 did not change significantly, and mitofusin 2 (Mfn2) showed a downward trend. Additionally, EA treatment significantly increased Drp1 and Parkin expression ($P < 0.05$) and partially increased Mfn2 expression (Fig. 7a). Moreover, after cerebral I/R, EA treatment promoted the translocation of Parkin and LC3 from the cytoplasm to mitochondria (Fig. 7b). Immunofluorescence also revealed that LC3 translocated to mitochondria marked by TOMM20 in the EA group (Fig. 7c). In addition, when Mdivi-1 (a selective inhibitor of mitophagy activation) was added, EA treatment alleviated the accumulation of damaged mitochondria after cerebral I/R was blocked (Fig. 7d and e). Mdivi-1 also further reduced the number of Nissl$^+$ neurons and aggravated neuronal injury, especially in the CA1 region of the hippocampus ($P < 0.05$, Fig. 7f), thereby eliminating the therapeutic effects of EA treatment on cerebral I/R injury. These results taken together suggest that EA increases Pink1/Parkin-mediated mitochondrial clearance in cerebral I/R.

4. Discussion

In this study, we first illustrated that EA improved cerebral infarct size, neurological function and neuronal damage in cerebral I/R. RNA-Seq results demonstrated that EA effects were closely related to mitochondrial function, ALP and mitophagy. Then, we observed mitochondrial function damage and damaged mitochondria accumulation during cerebral I/R and found that both were improved by EA, which ameliorated mitochondrial function damage by inhibiting nitro/oxidative stress. Finally, we demonstrated that EA improved damaged mitochondria accumulation by the PI3K/Akt/mTOR-induced ALP pathway, especially Pink1/Parkin-mediated mitophagy clearance, to protect against cerebral I/R injury.

The choice of acupoints is one of the key determinants of whether acupuncture will obtain curative effects. A literature review and our previous studies showed that combining “Baihui” (GV20) and “Zusanli” (ST36) had synergistic effects on alleviating neuronal injury caused by cerebral ischemia and was superior to other acupoint combinations [16–18]. In addition, GV20, which is located at the top of the head, is closely connected with the brain and is a key acupoint for regulating brain function. ST36, which is located at the knee joint in the lower extremity, can improve limb movement dysfunction induced by brain ischemia. This combination has both local and remote therapeutic effects. EA, when applied in combination with acupuncture and modern technology, is more suitable for experimental operations because it allows objective quantification and fewer influences by the surgeon [19]. In this study, we viewed ischemic stroke as a type of acute disease, and moderate-intensity stimulation is needed during the acute phase. In

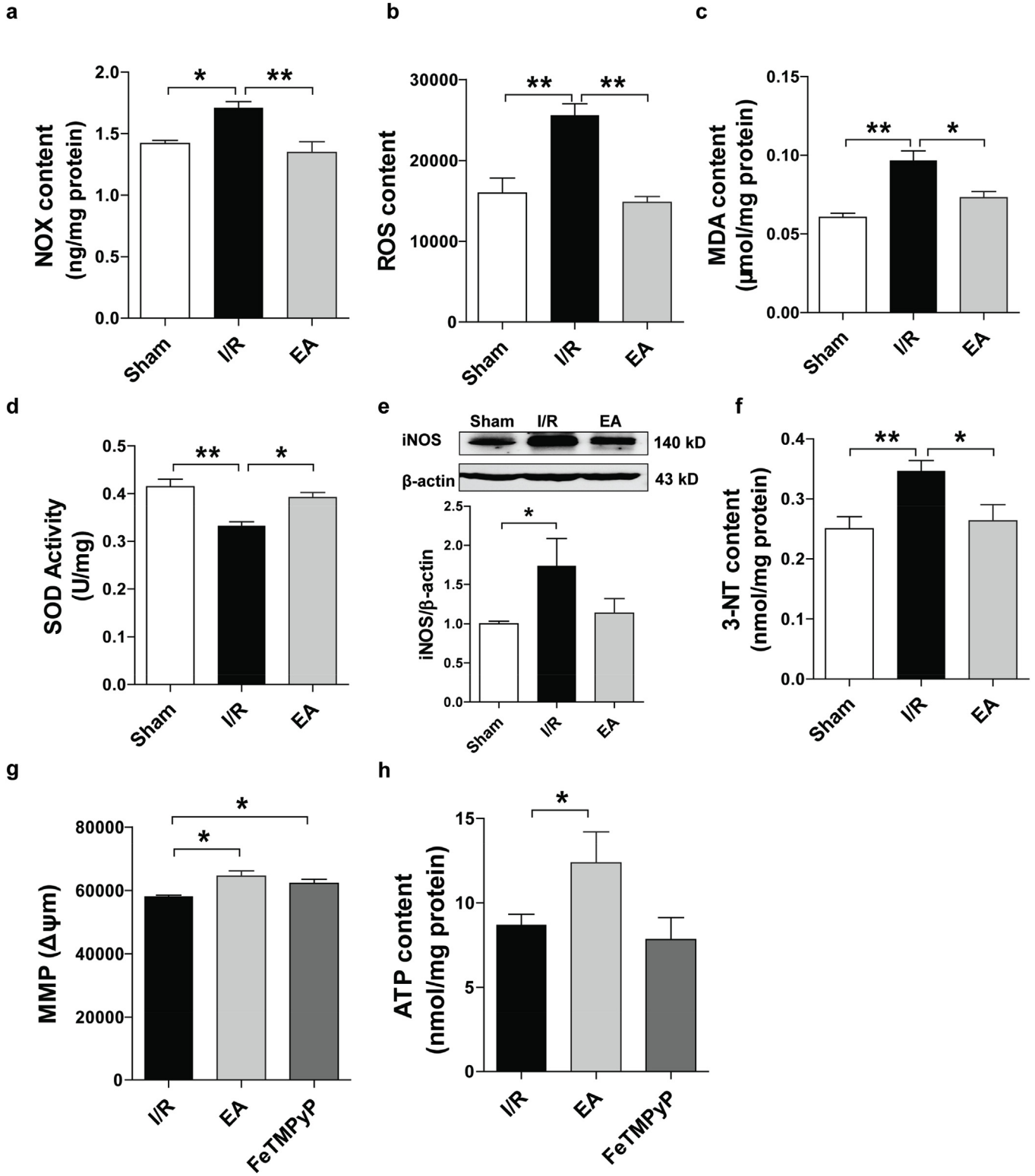

Fig. 5. EA ameliorated nitro/oxidative stress-induced mitochondrial injury in cerebral I/R. (a, b and c) The contents of NOX, ROS and MDA in the hippocampus in the ischemic area, n = 5 each. (d) SOD activity in each experimental group, n = 5 each. (e) The expression of iNOS by Western blot, n = 5 each. (f) The level of 3-NT of the hippocampus in the ischemic area, n = 5 each. (g) Mitochondrial membrane potential (MMP) assessed using a JC-1 fluorescence probe, n = 5 each. (h) The level of ATP in each experimental group, n = 5. Data are expressed as the mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$.

addition, the model of cerebral ischemia used in this study was established in rats rather than mice. Given these reasons, 20 Hz electrical stimulation with compressional waves was performed for 30 min as the treatment method. Our results show that EA improved cerebral infarct size (TTC staining), neurological function (Garcia JH scoring) and neuronal damage (Nissl staining) in cerebral I/R. Therefore, the purpose of our follow-up experiments was to identify the specific pathways and mechanisms of EA treatment.

Next-generation high-throughput RNA sequencing (RNA-Seq) is a technology that can objectively describe all gene expression changes in

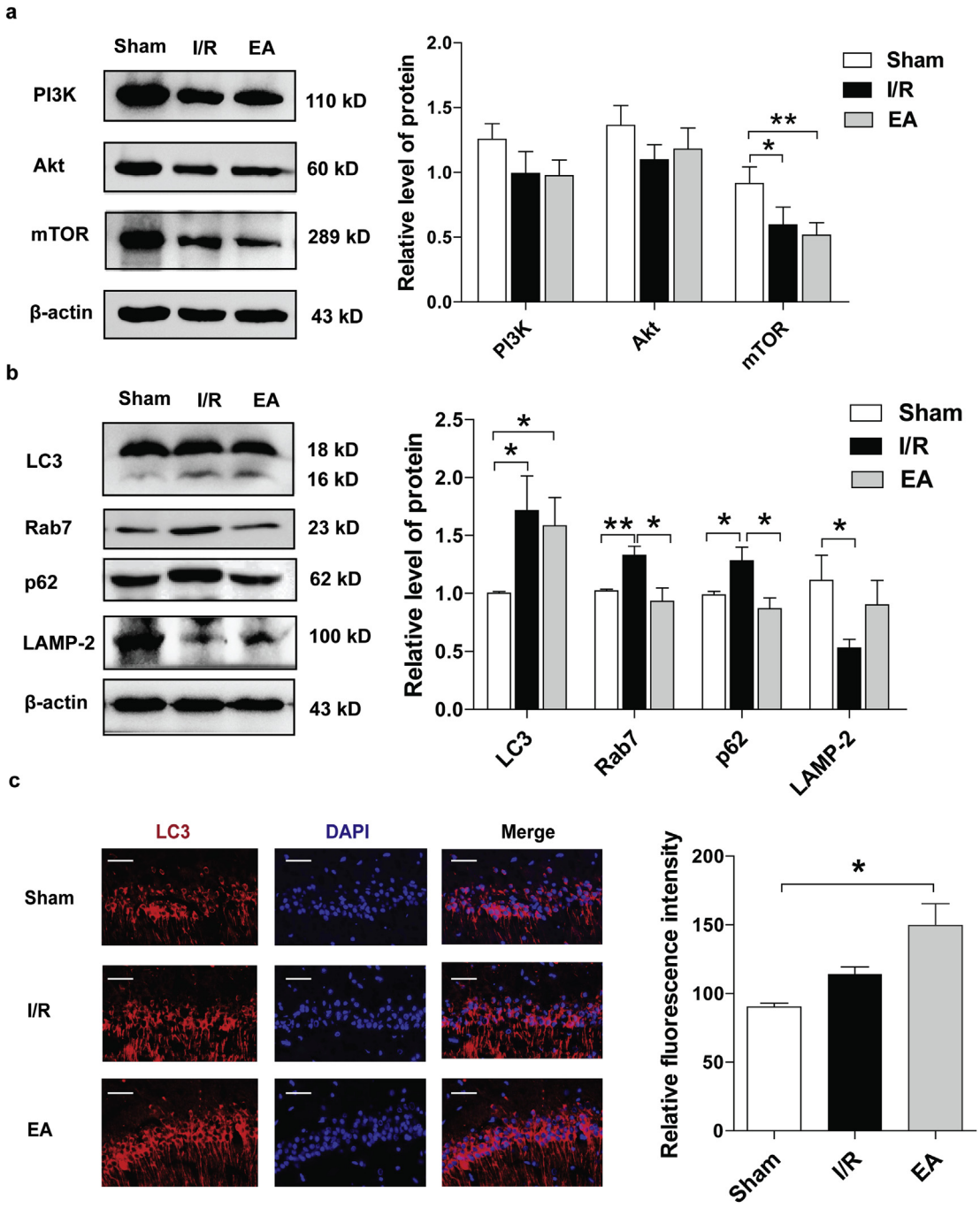

Fig. 6. EA improved PI3K/Akt/mTOR-mediated ALP dysfunction in cerebral I/R. (a) The expression of PI3K, Akt and mTOR by Western blot, n = 5 each. (b) The expression of LC3, Rab7, p62 and LAMP-2 by Western blot, n = 5 each. (c) The expression of LC3 in the CA1 region of the hippocampus by immunofluorescence, scale bar = 50 μm, n = 5 each. Data are expressed as the mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$.

diseases and reveal unknown transcripts that are not annotated in current databases [20]. In this study, we found that three significant pathways (phagosomes, the PI3K/Akt signaling pathway and metabolic pathways) were affected in cerebral I/R by EA treatment. PI3K/Akt are key regulatory factors that are affected in cerebral I/R and can enhance the survival of nerve cells, activate multiple protein kinases and play roles in the process of oxidative phosphorylation [21]. mTOR, the main downstream target of the PI3K/Akt signaling pathway, plays an important role in autophagy activation and the formation of early autophagosomal precursor structures [22]. Autophagosomes and phagosomes are similar in morphology and structure and mature into autolysosomes and phagolysosomes, respectively [23]. Autophagy and phagocytosis are both involved in cellular metabolism and generate essential nutrients and energy during times of starvation, as demonstrated by our results. Based on the above three findings, our subsequent experiments focused on mitochondria (the oxidative energy metabolism center of the cell) and autophagy/mitophagy.

The mPTP opens during persistent stimulation (such as I/R damage), damaging the permeability barrier of the mitochondrial membrane and leading to mitochondrial swelling, dissipation of MMP, the depletion of ATP and a transient increase of ROS [24]. CsA can block mPTP opening and exerted a protective effect on stroke in rats by maintaining the stability of the mitochondrial membrane to reverse neuronal injury [25]. Our results indicate that there was mitochondrial

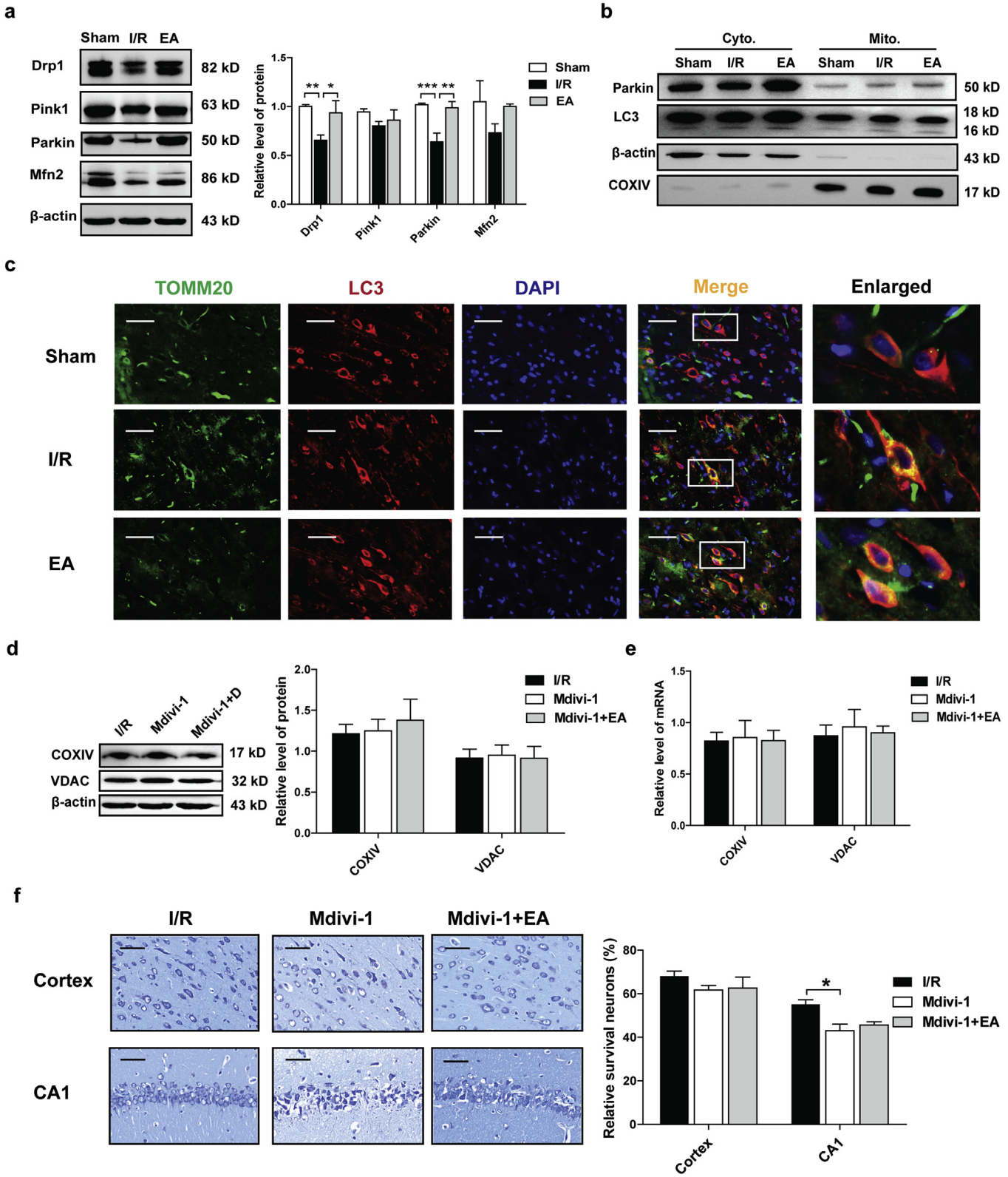

Fig. 7. EA increased Pink1/Parkin-mediated damaged mitochondria clearance in cerebral I/R. (a) The expression of Drp1, Pink1, Parkin and Mfn2 by Western blot, n = 5 each. (b) The expression of Parkin and LC3 in cytosolic and mitochondrial fractions by Western blot, n = 3 each. (c) Images of tissues co-stained for the mitochondrial markers TOMM20 (green), LC3 (red) and nuclear (blue), scale bar = 50 μm, n = 3 each. (d) The expression of mitochondrial markers (COX IV and VDAC) by Western blot, n = 5 each. (e) The mRNA levels of *COX IV*, *VDAC* and *TOMM20* by real-time PCR, n = 5 each. (f) The relative number of positive neurons by Nissl staining in the cortex and CA1 region of the hippocampus, n = 3 each, scale bar = 50 μm. Data are expressed as the mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

damage (decreased MMP and ATP) and an accumulation of damaged mitochondria (upregulated COX IV and TOMM20) in cerebral I/R. EA can maintain the stability of mitochondrial structures and reduce the accumulation of damaged mitochondria. In mitochondrial damage, excessive oxidative stress causes the most serious damage to the mitochondrial membrane structure, especially the inner membrane. Oxidative stress includes increased ROS and the loss of antioxidant molecules, such as SOD. NADPH oxidase (NOX) is considered to be a main source of brain tissue $O_2^{\cdot-}$ production during stroke. Other downstream effects of $O_2^{\cdot-}$ include the formation of $ONOO^-$ (a reaction product of NO and $O_2^{\cdot-}$). $ONOO^-$ exerts powerful biological effects and can induce DNA damage and lipid peroxidation and alter the function of proteins by nitration [26]. Our results indicate that EA reduced nitro/oxidative levels (i.e., decreased NOX, ROS, MDA and $ONOO^-$ content and increased SOD activity) and FeTMPyP (a type of $ONOO^-$ scavenger) improved mitochondrial damage (increased MMP). In summary, the above results indicate that EA reduces mitochondrial damage through anti-nitro/oxidative stress in cerebral I/R. However, the problem of mitochondrial accumulation requires further study, and the regulation of autophagy/mitophagy by EA was therefore further explored.

The ALP is responsible for the degradation and recycling of damaged or dysfunctional cytoplasmic proteins and intracellular organelles [27]. PI3K/Akt/mTOR are the key regulators that induce autophagy and regulate autophagosome formation. In addition, several proteins regulate the maturation of autophagosomes. LC3 is a typical protein marker of autophagosomes. After autophagosomes mature, they fuse with lysosomes because of an interaction between LC3 and Rab7, which is necessary for the formation of autolysosomes [28]. LAMP-2 is a lysosomal structural protein, and when autophagy flux is impaired, the cargo (including autophagy receptor p62/SQSTM1) is not degraded by lysosomes, resulting in the accumulation of p62/SQSTM1 [27]. Our results indicate that the autophagy process is generally activated during cerebral I/R; this process includes the inhibition of the PI3K/Akt/mTOR signaling pathway, the formation and maturation of autophagosomes (by upregulated LC3 and Rab7), and a reduction in the degradation of autolysosomes (including increased p62/SQSTM1 and decreased LAPMP-2 levels). EA can significantly ameliorate ALP dysfunction and improve autophagy clearance. Mitophagy is a process that specifically and selectively removes damaged mitochondria; it is mediated by the Pink1/Parkin pathway [5]. Before mitophagy occurs, damaged mitochondria undergo fission/fragmentation via a Drp1-mediated process and Mfn2 is a Parkin receptor for culling damaged mitochondria during mitophagy [15,29]. Mdivi-1 can inhibit mitophagy and prevent damaged or defective mitochondria clearance. Our results indicate that there is a deficiency in Pink1/Parkin-mediated mitophagy, including decreased Drp1, Parkin and Mfn2 levels. EA can increase Drp1, Parkin and Mfn2 levels and promote the translocation of Parkin and LC3 from the cytoplasm to mitochondria. Additionally, Mdivi-1 eliminated the therapeutic effects of EA treatment on cerebral I/R injury. Overall, the above results show that PI3K/Akt/mTOR-induced ALP dysfunction and deficiency in Pink1/Parkin-mediated mitophagy clearance occurs in cerebral I/R. EA can improve damaged mitochondria accumulation via Pink1/Parkin-mediated mitophagy clearance to protect against neuronal injury in cerebral I/R.

The accumulation of damaged mitochondria induced by nitro/oxidative stress is an important cause of cerebral I/R injury, and one way to reduce these injuries is to lower the number of damaged mitochondria. Therefore, autophagy activation has significant therapeutic potential in cerebral I/R injury [4]. In the case of impaired autophagy clearance or degradation, upstream autophagy induction may lead to the massive accumulation of autophagosomes, which may lead to toxic consequences. EA can directly target lysosomal function or enhance the entire pathway (including Pink1/Parkin-mediated mitophagy), thereby preventing a negative outcome. From a therapeutic perspective, EA-induced autophagy is a promising target mechanism. In many cases, it strengthens the removal of the damaged mitochondria that cause cerebral I/R injury, thus acting on the root causes of these injuries. Compared to simply inhibiting mTOR, EA presents substantial benefits. In particular, its long-term use is not associated with obvious side effects.

5. Conclusion

EA can ameliorate nitro/oxidative stress-induced mitochondrial function damage and improve damaged mitochondria accumulation via Pink1/Parkin-mediated mitophagy clearance to protect against neuronal injury in cerebral ischemia-reperfusion.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.07.004.

Conflicts of interest

None.

References

[1] L. Catanese, J. Tarsia, M. Fisher, Acute ischemic stroke therapy overview, Circ. Res. 120 (2017) 541–558.
[2] J.L. Yang, S. Mukda, S.D. Chen, Diverse roles of mitochondria in ischemic stroke, Redox Biol 16 (2018) 263–275.
[3] F. Liu, J. Lu, A. Manaenko, J. Tang, Q. Hu, Mitochondria in ischemic stroke: new insight and implications, Aging Dis 9 (2018) 924–937.
[4] F.M. Menzies, A. Fleming, A. Caricasole, C.F. Bento, S.P. Andrews, A. Ashkenazi, et al., Autophagy and neurodegeneration: pathogenic mechanisms and therapeutic opportunities, Neuron 93 (2017) 1015–1034.
[5] D.P. Narendra, S.M. Jin, A. Tanaka, D.F. Suen, C.A. Gautier, J. Shen, et al., PINK1 is selectively stabilized on impaired mitochondria to activate Parkin, PLoS Biol. 8 (2010) e1000298.
[6] F. Koyano, K. Okatsu, H. Kosako, Y. Tamura, E. Go, M. Kimura, et al., Ubiquitin is phosphorylated by PINK1 to activate parkin, Nature 510 (2014) 162–166.
[7] M. Komatsu, S. Waguri, T. Chiba, S. Murata, J. Iwata, I. Tanida, et al., Loss of autophagy in the central nervous system causes neurodegeneration in mice, Nature 441 (2006) 880–884.
[8] T. Hara, K. Nakamura, M. Matsui, A. Yamamoto, Y. Nakahara, R. Suzuki-Migishima, et al., Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice, Nature 441 (2006) 885–889.
[9] G. Kroemer, G. Marino, B. Levine, Autophagy and the integrated stress response, Mol. Cell 40 (2010) 280–293.
[10] X. Zhang, H. Yan, Y. Yuan, J. Gao, Z. Shen, Y. Cheng, et al., Cerebral ischemia-reperfusion-induced autophagy protects against neuronal injury by mitochondrial clearance, Autophagy 9 (2013) 1321–1333.
[11] S. Zhang, B. Wu, M. Liu, N. Li, X. Zeng, H. Liu, et al., Acupuncture efficacy on ischemic stroke recovery: multicenter randomized controlled trial in China, Stroke 46 (2015) 1301–1306.
[12] Q. Wang, Y. Peng, S. Chen, X. Gou, B. Hu, J. Du, et al., Pretreatment with electroacupuncture induces rapid tolerance to focal cerebral ischemia through regulation of endocannabinoid system, Stroke 40 (2009) 2157–2164.
[13] T. Tian, Y. Sun, H. Wu, J. Pei, J. Zhang, Y. Zhang, et al., Acupuncture promotes mTOR-independent autophagic clearance of aggregation-prone proteins in mouse brain, Sci. Rep. 6 (2016) 19714.
[14] J.Q. Kwong, J.D. Molkentin, Physiological and pathological roles of the mitochondrial permeability transition pore in the heart, Cell Metabol. 21 (2015) 206–214.
[15] M. Song, K. Mihara, Y. Chen, L. Scorrano, G.N. Dorn, Mitochondrial fission and fusion factors reciprocally orchestrate mitophagic culling in mouse hearts and cultured fibroblasts, Cell Metabol. 21 (2015) 273–286.
[16] L.M. Chavez, S.S. Huang, I. MacDonald, J.G. Lin, Y.C. Lee, Y.H. Chen, Mechanisms of acupuncture therapy in ischemic stroke rehabilitation: a literature review of basic studies, Int. J. Mol. Sci. 18 (2017).
[17] H. Xu, Y.M. Zhang, H. Sun, S.H. Chen, Y.K. Si, Electroacupuncture at GV20 and ST36 exerts neuroprotective effects via the EPO-Mediated JAK2/STAT3 pathway in cerebral ischemic rats, Evid Based Complement Alternat Med 2017 (2017) 6027421.
[18] H. Xu, Y. Zhang, H. Sun, S. Chen, F. Wang, Effects of acupuncture at GV20 and ST36 on the expression of matrix metalloproteinase 2, aquaporin 4, and aquaporin 9 in

rats subjected to cerebral ischemia/reperfusion injury, PLoS One 9 (2014) e97488.
[19] W. Zhu, Y. Ye, Y. Liu, X.R. Wang, G.X. Shi, S. Zhang, et al., Mechanisms of acupuncture therapy for cerebral ischemia: an Evidence-Based review of clinical and animal studies on cerebral ischemia, J. Neuroimmune Pharmacol. 12 (2017) 575–592.
[20] C. Trapnell, B.A. Williams, G. Pertea, A. Mortazavi, G. Kwan, M.J. van Baren, et al., Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation, Nat. Biotechnol. 28 (2010) 511–515.
[21] L.A. Jansen, G.M. Mirzaa, G.E. Ishak, B.J. O'Roak, J.B. Hiatt, W.H. Roden, et al., PI3K/AKT pathway mutations cause a spectrum of brain malformations from megalencephaly to focal cortical dysplasia, Brain 138 (2015) 1613–1628.
[22] G. Tang, K. Gudsnuk, S.H. Kuo, M.L. Cotrina, G. Rosoklija, A. Sosunov, et al., Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits, Neuron 83 (2014) 1131–1143.
[23] V. Deretic, Autophagosome and phagosome, Methods Mol. Biol. 445 (2008) 1–10.
[24] T. Xu, W. Ding, X. Ao, X. Chu, Q. Wan, Y. Wang, et al., ARC regulates programmed necrosis and myocardial ischemia/reperfusion injury through the inhibition of mPTP opening, Redox Biol 20 (2018) 414–426.
[25] F. Fakharnia, F. Khodagholi, L. Dargahi, A. Ahmadiani, Prevention of cyclophilin D-Mediated mPTP opening using Cyclosporine-A alleviates the elevation of necroptosis, autophagy and Apoptosis-Related markers following global cerebral Ischemia-Reperfusion, J. Mol. Neurosci. 61 (2017) 52–60.
[26] H. Wang, Q. Sun, Y. Zhou, H. Zhang, C. Luo, J. Xu, et al., Nitration-mediated deficiency of cystathionine beta-synthase activity accelerates the progression of hyperhomocysteinemia, Free Radic. Biol. Med. 113 (2017) 519–529.
[27] Y. Liu, X. Xue, H. Zhang, X. Che, J. Luo, P. Wang, et al., Neuronal-targeted TFEB rescues dysfunction of the autophagy-lysosomal pathway and alleviates ischemic injury in permanent cerebral ischemia, Autophagy (2018) 1–17.
[28] Y.C. Wong, D. Ysselstein, D. Krainc, Mitochondria-lysosome contacts regulate mitochondrial fission via RAB7 GTP hydrolysis, Nature 554 (2018) 382–386.
[29] Y. Chen, G.N. Dorn, PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria, Science 340 (2013) 471–475.