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RESEARCH

Mito-Apocynin Protects Against Kainic Acid-Induced Excitotoxicity by Ameliorating Mitochondrial Impairment

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Abstract

Neurodegenerative diseases represent significant global health challenges, with rising incidence rates. A substantial body of evidence indicates that excitotoxicity may be a critical target in the context of these diseases. However, effective pharmacological interventions aimed at mitigating excitotoxicity remain elusive. This study aimed to elucidate the neuroprotective effects and mechanisms of the mitochondrion-targeted NOX inhibitor, mito-apocynin, in the context of kainic acid (KA)-induced excitotoxicity. Our findings demonstrate that KA disrupts mitochondrial morphology, leading to impaired energy metabolism and mitochondrial dysfunction. Western blotting experiments revealed that KA compromises mitochondrial quality control. Additionally, Nissl staining and CCK8 assays indicated that mito-apocynin (administered at 75 μg/kg *in vivo* and 1 μM *in vitro*) significantly reduced neuronal death resulting from KA-induced excitotoxic damage in both *in vivo* and *in vitro* models. Furthermore, mito-apocynin improved neurobehavioral deficits induced by KA and mitigated mitochondrial dysfunction observed *in vitro*. Notably, mito-apocynin significantly reversed the KA-induced increase in NOX4 levels within the striatal mitochondria, reduced the ratio of phosphorylated DRP1 (Ser616) to total DRP1, and enhanced the expression of PGC-1α, PINK1, and Parkin proteins throughout the total striatum. In summary, mito-apocynin alleviates oxidative stress, preserves normal mitochondrial function and energy metabolism, and promotes mitochondrial quality control by modulating NOX expression in mitochondria, thereby reducing KA-induced excitotoxic damage.

Keywords Mito-apocynin · KA · Excitotoxicity · Mitochondrial dysfunction · NADPH oxidase

Introduction

Neurodegenerative diseases represent significant global health challenges, with rising incidence rates [1, 2]. Ischemic stroke is believed to result from excitotoxicity induced by the overactivation of glutamate receptors [3, 4]. A substantial body of evidence indicates that excitotoxicity may serve as a potential target in the context of neurodegenerative diseases [5, 6]. The dysregulation of neuronal calcium homeostasis, oxidative stress, and mitochondrial dysfunction can arise from excessive glutamate release or impaired uptake [6–9]. Excitotoxicity is a defining characteristic of cellular responses to stress that occur during hypoxic/ischemic injuries and in diseases associated with neurodegeneration [10, 11]. However, there is a notable absence of effective pharmacological interventions aimed at mitigating excitotoxicity. Thus, it is imperative to investigate the molecular mechanisms underlying neuronal damage caused by excitotoxicity to explore new strategies for the prevention and treatment of neurodegenerative diseases [12].

Studies have shown that there are KA receptors on the medium spiny neurons of the striatum, particularly in the projection fibers from the anterior cingulate cortex to the dorsal striatum, where a large number of direct projection fibers are present [13]. We successfully modeled neuroexcitatory damage through KA injection in the right striatum of the mouse brain, without affecting the left striatum or cerebral cortex. Supplementation with NADPH provided protection to neurons against KA-induced excitotoxic injury [14, 15]. However, NADPH exhibits a dual role; in addition to functioning as an antioxidant, it serves as a substrate for

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NADPH oxidase (NOX), an enzyme that generates reactive oxygen species (ROS) by oxidizing NADPH [16, 17]. Three primary NOX isoforms are expressed in the central nervous system: NOX1, NOX2, and NOX4 [18]. Our findings indicate that following exposure to excitotoxins, NOX4 expression was significantly elevated [15]. The production of NOX is intricately linked to ROS, positioning it as a promising target for novel treatments of neurodegenerative diseases [1, 18, 19]. Various neurodegenerative diseases are associated with increased NOX activity and expression [20]. Furthermore, NOX-mediated superoxide generation is a critical mechanism underlying excitotoxic cell death resulting from NMDA receptor activation [21].

ROS are primarily generated by NOX and mitochondria [22]. ROS produced by NOX can induce damage to mitochondria [23]. Conversely, in certain instances, ROS generated by mitochondria may also stimulate NOX activity [24]. This interplay, or crosstalk, between mitochondria and NOX may lead to an excessive production of ROS, presenting a potential new target for pharmacological intervention [25, 26]. Recent studies have revealed that NOX4 is localized within mitochondria, prompting increased interest in the interactions between NOX4 and mitochondrial function [27]. Historically, the activity of the mitochondrial respiratory chain was regarded as the primary source of ROS within cells [28]. However, contemporary research indicates that the respiratory chain may function more as a target of ROS damage rather than the principal source of ROS production. It is posited that mitochondrial NOX4 serves as a central mediator of oxidative stress. Mitochondrial NOX4 has been identified as a significant source of superoxide in neuronal mitochondria [29, 30]. Furthermore, NOX4 plays a crucial role in regulating mitochondrial energy metabolism, biogenesis, and mitochondrial DNA repair in the context of cardiovascular diseases [31]. Therefore, it is essential to investigate the variations in mitochondrial NOX levels during excitotoxicity and the role of mitochondrial NOX in this process.

The objective of this study was to evaluate whether the mitochondrion-targeted NOX inhibitor mito-apocynin offers protection against KA-induced excitotoxicity and to assess its impact on mitochondrial damage resulting from excitotoxicity.

Materials and Methods

Animals and Drug Administration

SPF-grade Institute of Cancer Research (ICR) male mice, 25–30 g, were purchased from ZhaoYan (Suzhou) Company. SPF animal centers maintained constant temperatures of 22 °C and a humidity of 50%−60% for the rearing of mice. Institutional regulations on animal health apply to the use of these animals. In all cases, Soochow University's Institutional Animal Care and Use Committee approved the protocols.

Stereotaxic apparatuses were used to fix the mice after anesthesia. The right striatum was injected with 0.625 nmol of KA (Sigma Aldrich, K0250). Coordinates of the right striatum: 0.8 mm anterior to the bregma, 1.8 mm lateral to the sagittal suture, and 3.5 mm ventral to the pial surface. The injection volume was 1 µl, and the injection speed was 0.5 µl/min. Mito-apocynin (HY-135869, MCE, ig) was administered once daily, starting one day before KA injection and continuing for the duration of the experiment.

Isolation of Tissue Mitochondrial and Cytoplasmic Fractions

After the mice were sacrificed, the striatum was isolated. A tenfold volume of Mitochondrial Isolation Reagent A solution (Beyotime Biotechnology, C3606) with 1% PMSF (Beyotime Biotechnology, ST505) was added and homogenized 30 times. Five minutes of centrifugation at 600 × g was performed on the tissue homogenate. The supernatant was collected for the isolation of mitochondria. In the supernatant, 10 min of centrifugation were carried out at 11,000 × g. This supernatant was aspirated as a cytoplasmic fraction. The precipitate was resuspended by adding 100 µl of Mitochondrial Isolation Reagent A solution. Afterward, it was centrifuged for ten minutes at 11,000 × g. The resuspension was transferred to an EP tube containing 22%/55% Percoll and centrifuged at 20,000 × g for 20 min. The white flocculent between the Percoll gradient was the fraction of mitochondria.

Western Blotting

By our previous description, Western blotting was performed [14]. Antibody source: NOX1 antibody (Proteintech, 17772–1-AP); NOX2 antibody (Santa Cruz, sc-130543); NOX4 antibody (Abcam, ab109225); DRP1 antibody (Cell Signaling Technology, 5931S); DRP1 (phospho-ser616) antibody (SAB, 12749); PINK1 antibody (Absin, abs100425); Parkin antibody (Santa Cruz, sc-2282); PGC-1α antibody (Santa Cruz, sc-3067); GAPDH antibody (Abcam, ab8245); β-actin antibody (Sigma Aldrich, A5441); α-Tubulin antibody (Abcam, ab7291); VDAC antibody (Cell Signaling Technology, 12454).

Transmission Electron Microscopy

Isolation of the striatum after heart perfusion. Preparation of 2 mm × 2 mm ultrathin tissue sections. Store at 4 °C in electron microscope fixative (Servicebio, G1102). Samples

were double-stained using lead-uranium. The copper mesh containing the samples was placed in a transmission electron microscope for observation.

Nissl Staining

Whole brains were isolated after heart perfusion and fixed in 4% paraformaldehyde. Sucrose was dehydrated, and coronal sections were prepared using a vibrating slicer. Nissl staining (Beyotime Biotechnology, C0117) was performed for 30 min. For 2 min, the sections were dehydrated and decolorized in 75%, 95%, and 100% ethanol. Xylene was used to permeabilize the slices for 10 min, followed by sealing with neutral resin. Neuronal morphology was observed under a microscope, and the number of normal neurons in the striatal center was counted at 20 × magnification.

Behavioral Test

We conducted a series of behavioral tests, including the cylinder test, adhesive removal test, and inverted grid test. These tests were used to evaluate the motor function and coordination of the mice. To ensure the accuracy and reproducibility of the behavioral results, we used a separate group of animals for behavioral analysis. These animals did not participate in Western blot analysis or other biochemical tests to avoid interference from experimental procedures. By using an independent group of animals, we were able to more accurately assess the effects of Mito-apocynin on KA-induced behavioral deficits.

Cylinder test: Mice were placed in a 10 cm diameter glass round cylinder. The number of unilateral and bilateral forelimb contacts with the cylinder wall was recorded within 3 min. The proportion of unilateral contact was counted.

Adhesive removal test: The mice were acclimated to their new environment before starting the experiment. A 0.2-inch piece of tape was applied to the nose of the mice. The time needed to remove the tape from the forelimbs of the mice was recorded for no more than 1 min.

Inverted grid test: A pretest was performed on the day of the experiment to acclimatize the mice to the test. The mice were placed in the center of a 15 cm^2 horizontal square grid. The grid was raised 20 cm from the table, and the grid was flipped up and down. The time that the mice remained on the grid was recorded for no more than 1 min.

Primary Neuron Culture

Primary cortical neurons were cultured in Neurobasal Medium (Gibco, 21103049) supplemented with 2% B27 Supplement (Gibco, 17504044), 50 U/mL penicillin–streptomycin (Beyotime, C0222), 1% glutamine (Gibco, 35050061). Separate the cerebral cortex of 18-day-old ICR mouse embryos. Add 2.5% trypsin and digest at 37 °C for 15 min. After termination of digestion, add DNAase and gently blow 100 times. Centrifuge at 200 × g for 5 min. Resuspend the cells by adding neuronal medium and filter the cells through a 40 μm cell sieve. Cell suspensions were diluted to 10^6 cells/ml and incubated at 37 °C with 5% CO_2. After incubation until the fourth day, cytosine arabinoside (at a final concentration of 10 μmol/L) was added to the culture medium to inhibit the growth of glial cells. The culture was continued until the seventh day, at which point it could be used for subsequent experiments.

Measurement of ATP Levels

The experiment was performed according to the instructions provided with the ATP assay kit (Beyotime Biotechnology, S0026). The cells were collected, and 50 μl of ATP assay lysate was added. Five minutes at 12,000 × g in a centrifuge. Collection of supernatants for ATP level determination. Place 100 μl of ATP assay working solution into each well in a black 96-well plate. This was allowed to sit at room temperature and was protected from light for 3–5 min. Add 20 μl of sample to the assay wells. RLU values were determined. The protein concentration was also measured by a BCA kit (Takara Bio, T9300A). The ATP level was expressed as nmol/mg.

Cell Viability

Ninety-six-well plates were used to inoculate primary neurons. Pretreatment with Mito-apocynin for 4 h was followed by treatment with 100 μM KA for 8 h. To measure cell viability, we used a kit called CCK-8 (DOJINDO, CK04). The reaction was carried out at 37 °C for 3 h. Microplate readers were used to measure absorbance at 450 nm.

Measurement of Mitochondrial Membrane Potential

The membrane potential of the mitochondria was detected using JC-1 (Beyotime Biotechnology, C2006). Primary cortical neurons were inoculated in a 24-well plate. We pretreated neurons with Mito-apocynin for 4 h and then treated them with 100 μM KA for 8 h. The medium was aspirated, and the cells were washed twice in HBSS. Working solution for JC-1 staining should be added in 0.3 ml. The mixture was incubated for 20 min at 37 °C in the dark. The cells were washed with JC-1 buffer 3 times. The cells were observed under a fluorescence microscope.

Measurement of Mitochondrial Superoxide Levels

Mitochondrial superoxide levels were detected using MitoSOX Red mitochondrial superoxide indicator (Yeasen,

40778ES50). Primary cortical neurons were inoculated in 24-well plates. Neurons were pretreated with Mito-apocynin for 4 h, followed by treatment with 100 μM KA for 8 h. The medium was aspirated, and the cells were washed twice with HBSS. Add 0.3 ml MitoSOX Red working solution and incubate for 10 min at 37 °C protected from light. The HBSS disinfection was repeated three times, and the cells were stained again with Hoechst's reagent in 0.3 ml. wash twice with HBSS. The cells were observed under a fluorescence microscope.

Statistical Analysis

Statistical analysis and graphing of experimental data were performed using GraphPad Prism 8. The mean and standard error of the experimental data are expressed as the mean ± SEM. Analysis of the differences between the two data groups was performed using an unpaired t-test, one-way analysis of variance for multigroup data was performed using one-way ANOVA, two-way ANOVA was used for multivariate analysis of differences in multiple groups of data, statistical significance was determined by $*P < 0.05$, and no */# in the statistical graph indicates no significant difference.

Results

KA-Induced Excitotoxicity Upregulated the Expression Level of NOX4 in Mitochondria

To investigate the relationship between the NOX family and excitotoxicity, we stereotactically injected the glutamate analogue KA into the right striatum of mice to establish an animal model of excitotoxicity. The control group received an equivalent volume of normal saline using the same method. We assessed the total protein levels of NOX1, NOX2, and NOX4 at various time points (6, 12, 24, 48, and 96 h) following KA treatment using Western blot analysis. Our findings revealed that KA upregulated the expression of NOX4 at 6 h and increased the expression of NOX2 at 48 and 96 h, while NOX1 expression remained unchanged (Fig. 1a-f). To further elucidate the expression of the NOX family within the mitochondrial compartment, we isolated mitochondria and cytosolic fractions. The results indicated a significant increase in mitochondrial NOX4 expression following KA injection, whereas cytoplasmic NOX4 expression showed no change (Fig. 2a-c). In contrast, the expression of NOX2 and NOX1 in mitochondria appeared unaffected by KA; however, KA did upregulate the expression of NOX2 in the cytoplasm (Fig. 2d-h). These results suggest that, in the KA-induced excitotoxicity model, mitochondrial function is primarily regulated by the upregulation of mitochondrial NOX4 expression.

KA-Induced Excitotoxicity Impairs Mitochondrial Morphology and Disturbs Quality Control Systems

The overactivation of glutamate receptors can lead to mitochondrial damage [32]. Using transmission electron microscopy, we observed that following KA treatment, the cytoplasmic matrix of mitochondrial neurons became lighter, the mitochondria swelled, the mitochondrial matrix also lightened, and the mitochondrial cristae appeared ruptured (Fig. 3a). The mitochondrial quality control system, which encompasses mitochondrial biogenesis, dynamics, and mitophagy, is essential for maintaining functional mitochondria. Therefore, we employed Western blotting to assess the effects of KA-induced excitotoxicity on the mitochondrial quality control system. PGC-1α, a key protein that regulates mitochondrial biogenesis [33], showed a significant increase in expression levels after KA injection for 24–48 h (Fig. 3b-c). Phosphorylated DRP1 (Ser616), which promotes mitochondrial fission, exhibited a decrease in the ratio of phosphorylated DRP1 (Ser616) to total DRP1 after KA injection for 12–48 h (Fig. 3d-e). PINK1 and Parkin work synergistically to enhance the autophagic clearance of damaged mitochondria [34], and after KA injection for 24 h, the expression levels of PINK1 and Parkin proteins significantly increased (Fig. 3f-i). These findings indicate that KA-induced excitotoxicity disrupts mitochondrial morphology and compromises the quality control system.

Mito-Apocynin Protects Striatal Neurons from KA-Induced Excitotoxicity

To investigate the potential role of NOX inhibitors in neuroprotection, we utilized Mito-apocynin, a mitochondria-targeted NOX inhibitor. This compound, Apocynin, binds to the cationic portion of the mitochondria-targeting moiety triphenylphosphonic acid, resulting in the formation of the mitochondrion-targeted NOX inhibitor Mito-apocynin [35, 36]. Given the high lipophilicity of the blood–brain barrier, Mito-apocynin is capable of crossing this barrier to reach the striatum [37]. Mito-apocynin was pre-administered to mice one day prior to KA injection. Following this, Mito-apocynin was administered once daily, and brain tissue was isolated for Nissl staining after a period of 14 days (Fig. 4a). Exogenous supplementation of Mito-apocynin at doses of 18.75 μg/kg, 37.5 μg/kg, and 75 μg/kg significantly ameliorated KA-induced neuronal wrinkling and loss of Nissl bodies (Fig. 4b). Furthermore, Mito-apocynin reduced the incidence of neuronal death in a dose-dependent manner (Fig. 4c). Consequently, we selected the 75 μg/kg dosage of

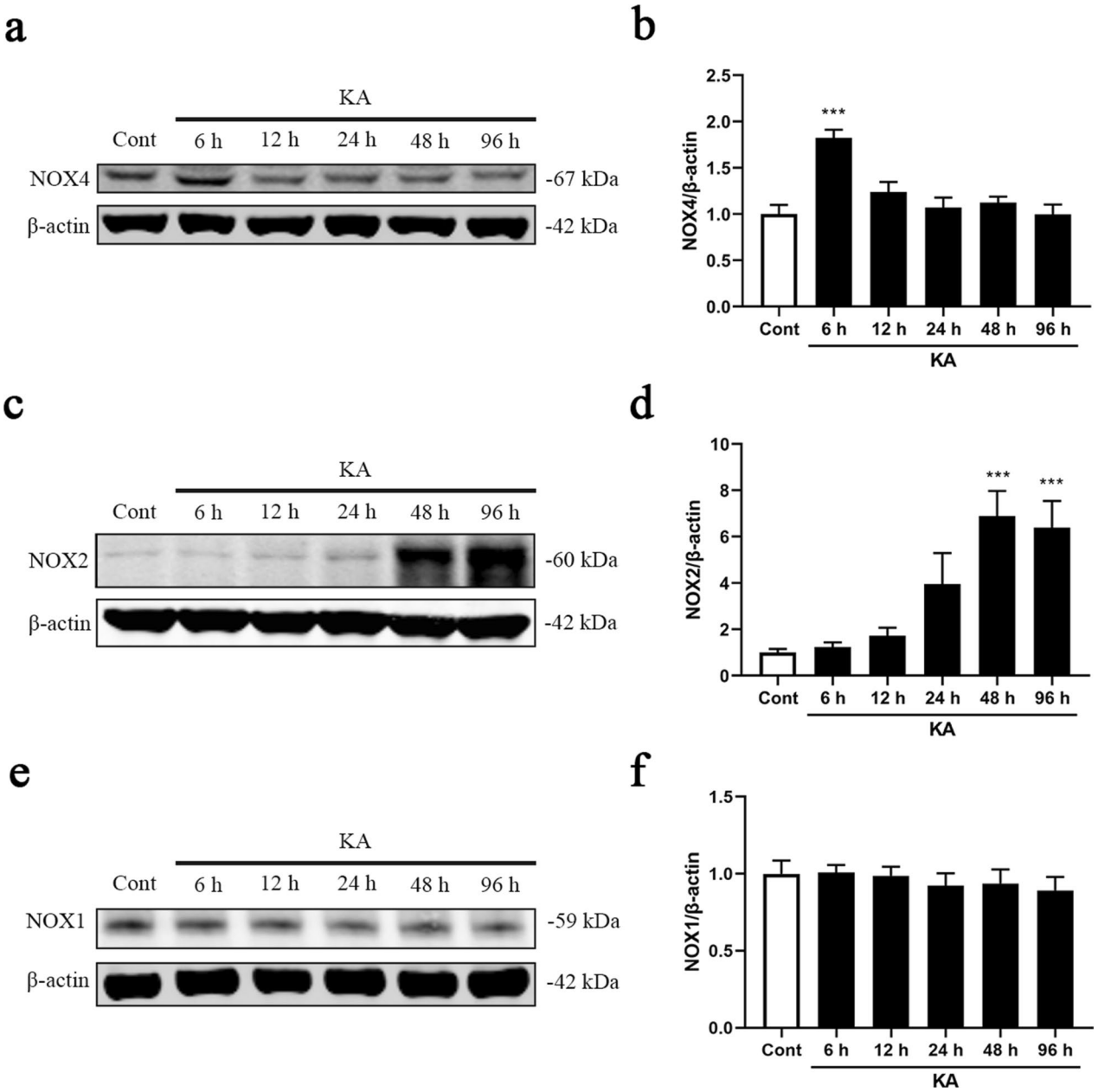

Fig. 1 NADPH oxidase subtype NOX4, NOX2 and NOX1 protein expression in KA-induced excitotoxicity. Mice were killed 6, 12, 24, 48 and 96 h after KA injection for western blotting. (**a**, **b**) Time course of KA-induced changes in NOX4 protein expression in the striatum. $n=4$. (**c**, **d**) Time course of KA-induced changes in NOX2 protein expression in the striatum. $n=6$. (**e**, **f**) Time course of KA-induced changes in NOX1 protein expression in the striatum. $n=6$. Data are expressed as the mean ± SEM. *** $P<0.001$ versus control, one-way ANOVA, Dunnett test

Mito-apocynin, which exhibited the most pronounced protective effect, for subsequent *in vivo* studies.

Mito-Apocynin Ameliorates KA-Induced Motor Behavioral Deficits in Mice

Striatal injury leads to alterations in motor and muscle control in mice, particularly affecting the forelimbs [38]. In a previous study, we demonstrated that KA-induced excitotoxicity results in motor dysfunction in mice [39]. Consequently, we conducted the cylinder test, the adhesive removal test, and the inverted grid test to assess striatal damage [40–42]. In the cylinder test, control mice tend to use both limbs to contact the wall of the container, while the proportion of mice in the KA-treated group that use a single limb to touch the wall increases. Treatment

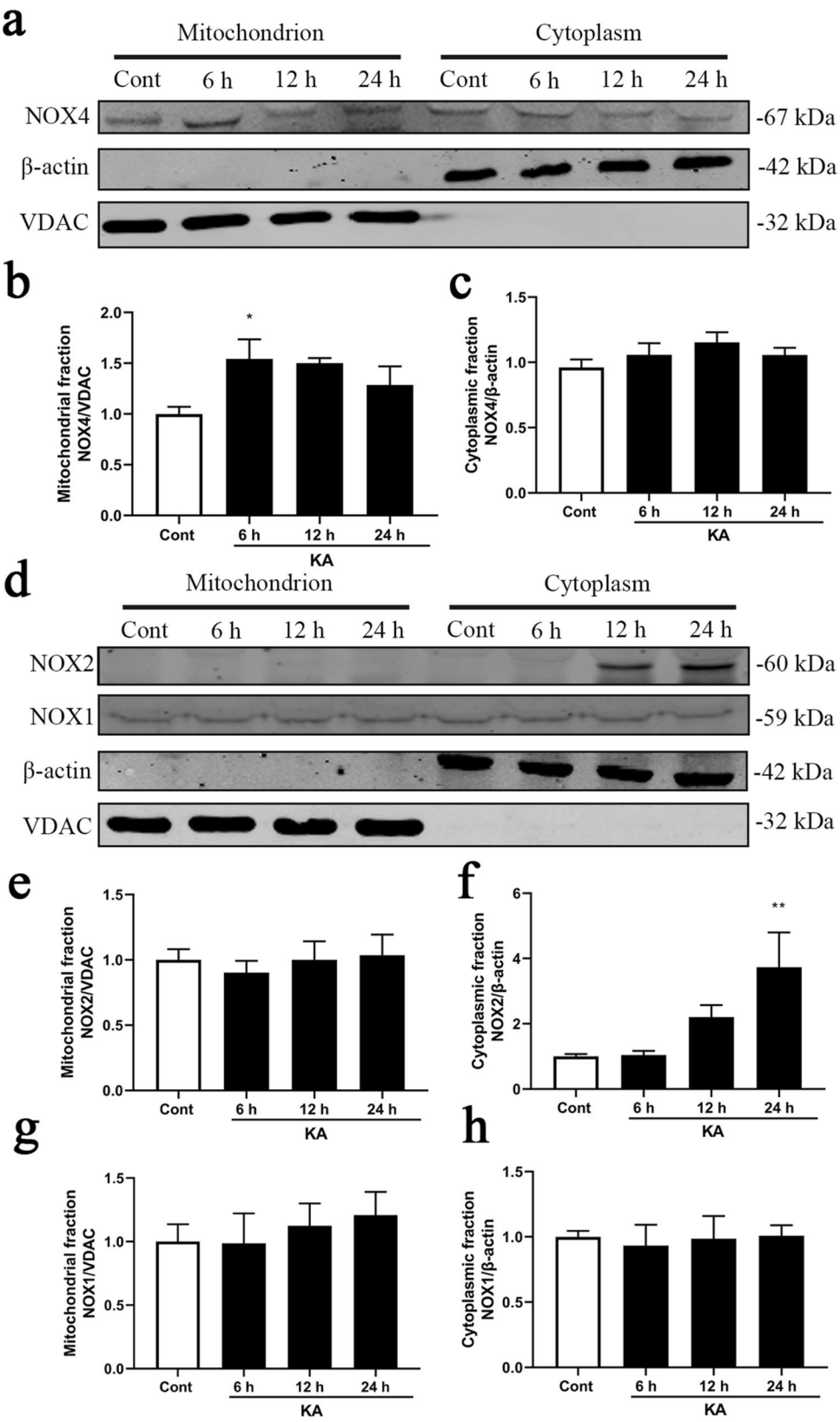

Fig. 2 Mitochondrial and cytoplasmic fraction NOX4, NOX2 and NOX1 protein expression in KA-induced excitotoxicity. Mice were killed 6, 12, and 24 h after KA injection, and then the mitochondrial and cytoplasmic fractions were isolated for western blotting. (**a**-**c**) Time course of KA-induced changes in NOX4 protein expression in the mitochondrial and cytoplasmic fractions of the striatum. $n=5$. (**d**) Time course of KA-induced changes in NOX2 and NOX1 protein expression in the mitochondrial and cytoplasmic fractions of the striatum. (**e**–**f**) Quantification of western blotting analysis of NOX2. $n=6$. (**g**-**h**) Quantification of western blotting analysis of NOX1. $n=3$. Data are expressed as the mean ± SEM. * $P<0.05$ versus control, ** $P<0.01$ versus control, one-way ANOVA, Dunnett test

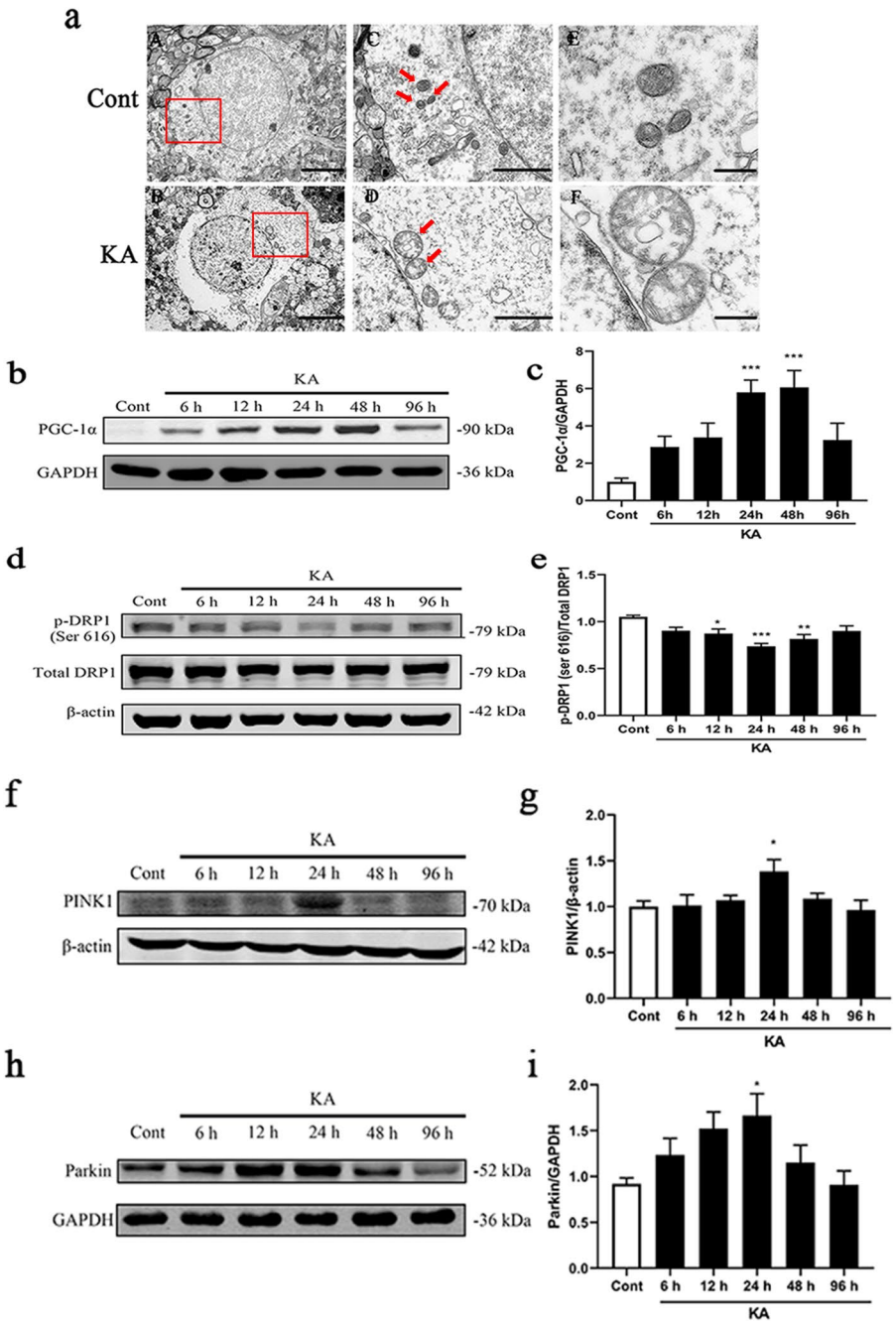

Fig. 3 KA-induced excitotoxicity impairs mitochondrial morphology and disturbs quality control systems. (**a**) Mice were killed 48 h after KA injection. Striatum tissue was collected for transmission electron microscopy**.** Representative electron microscopy micrographs of mitochondria in striatal neurons. Images C-D are representative areas indicated by the red wireframes in A-B. Images E–F show representative mitochondria indicated by red arrows in C-D. Scale bar = 5 μm in A-B; Scale bar = 2 μm in C-D; Scale bar = 100 nm in E–F. (**b**, **c**) Mice were killed 6, 12, 24, 48 and 96 h after KA injection for western blotting. Time course of KA-induced changes in PGC-1α in the striatum. $n = 6$. (**d**, **e**) Time course of KA-induced changes in phospho-DRP1 (Ser616)/total DRP1 in the striatum. $n = 4$. (**f**, **g**) Time course of KA-induced changes in PINK1 protein expression in the striatum. $n = 5$. (**h**, **i**) Time course of KA-induced changes in Parkin protein expression in the striatum. $n = 4$. Data are expressed as the mean ± SEM. * $P < 0.05$ versus control,** $P < 0.01$ versus control, *** $P < 0.001$ versus control, one-way ANOVA, Dunnett test

with Mito-apocynin promotes recovery of the forelimbs in mice, leading to a decrease in the proportion of single-limb wall contact (Fig. 5a). The adhesive removal test assesses the mice's ability to control fine motor movements. Control mice can remove the adhesive labels in a short period of time, whereas the time taken for mice in the KA-treated group to remove the adhesive labels significantly increases. Treatment with Mito-apocynin reduces the time required for mice to remove the adhesive labels (Fig. 5b). The inverted grid test evaluates the muscle tone of the mice's limbs. Control mice are less likely to fall off the grid and can maintain their position for a longer duration. In contrast, mice in the KA-treated group have difficulty staying on the grid. Treatment with Mito-apocynin increases the time that mice can remain on the grid (Fig. 5c). We performed an equivalent distribution for these three behavioral tests, assigning each test a score of 20 points to calculate the neurological deficit score, with a total possible score of 60 points. A lower score indicates more severe behavioral deficits in the mice. The results showed that Mito-apocynin could restore KA-induced motor behavioral dysfunction in mice (Fig. 5d).

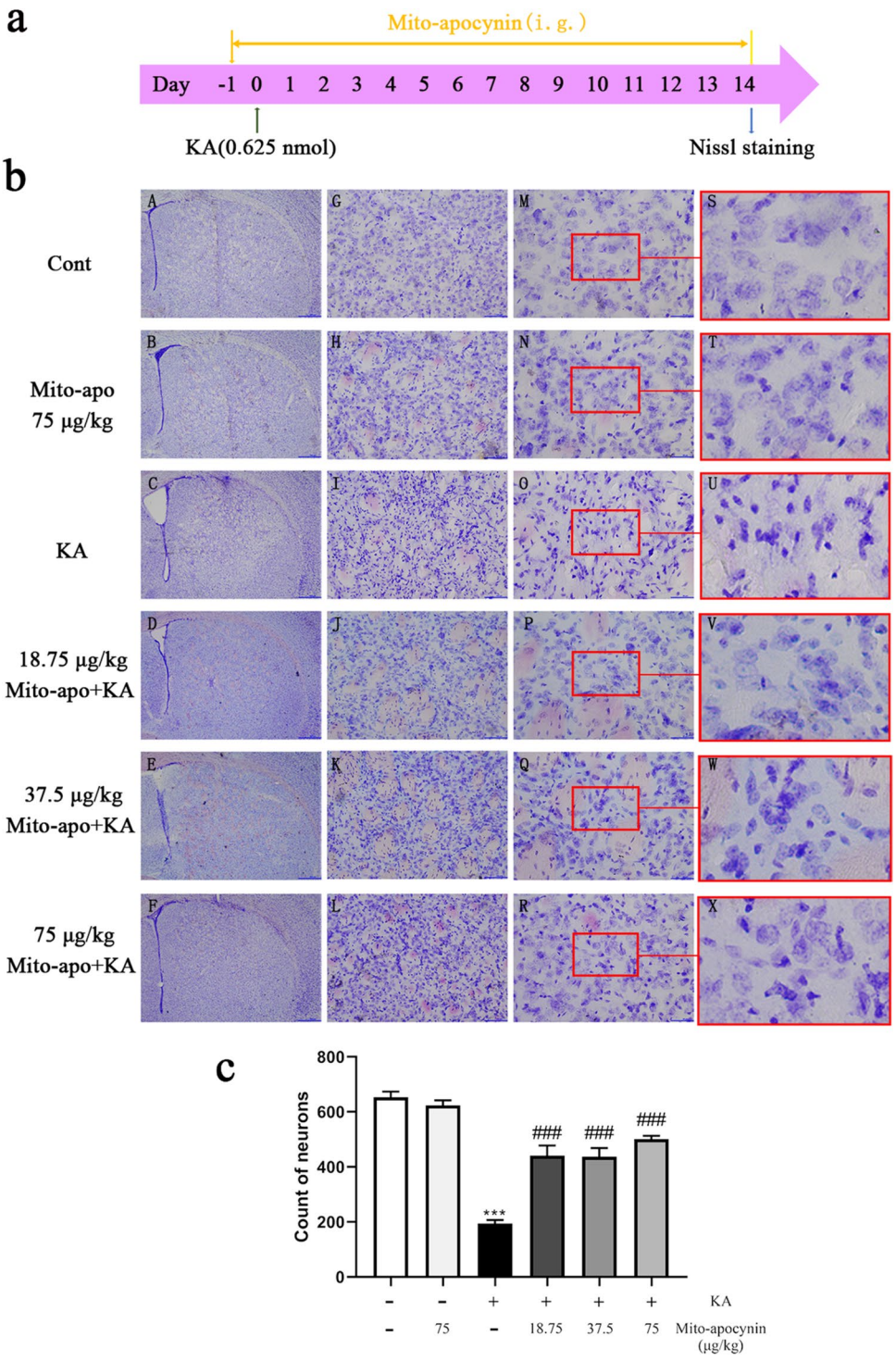

Fig. 4 Effects of Mito-apocynin on KA-induced excitotoxic injury *in vivo*. (**a**) Study road map of the animal experiment. Mice were administered 18.75 μg/kg, 37.5 μg/kg, and 75 μg/kg Mito-apocynin (i.g.) 1 day in advance. Unilateral injection of KA (0.625 nmol) was used to construct an animal model of excitotoxicity. Mito-apocynin was administered continuously for 14 days. Brain sections were stained with Nissl's stain. (**b**) Representative images were taken in the center of the drug injection. (Scale bar = 500 μm in A-F; Scale bar = 100 μm in G-L; Scale bar = 50 μm in M-R; and 20 μm in S-X) (**c**) Quantitative results of normal morphological neurons. $n = 3$. Data are expressed as the mean ± SEM. *** $P < 0.001$ versus control, ### $P < 0.001$ versus KA-treated group, one-way ANOVA, Tukey test

Mito-Apocynin Ameliorates KA-Induced Cytotoxicity and Mitochondrial Dysfunction

To investigate whether Mito-apocynin has a restorative effect on KA-induced mitochondrial damage *in vitro*, we conducted the experiment as depicted in Fig. 6a. Primary neurons were pre-treated with varying concentrations of Mito-apocynin (0.25, 0.5, 1, and 2 μM) for 4 h, followed by treatment with 100 μM KA for 8 h. Cell viability was assessed using the CCK8 assay, and the results indicated that Mito-apocynin could reverse the KA-induced decrease in cell viability and inhibit the cytotoxicity of KA in a dose-dependent manner (Fig. 6b). Consequently, in subsequent *in vitro* experiments, we selected a treatment dose

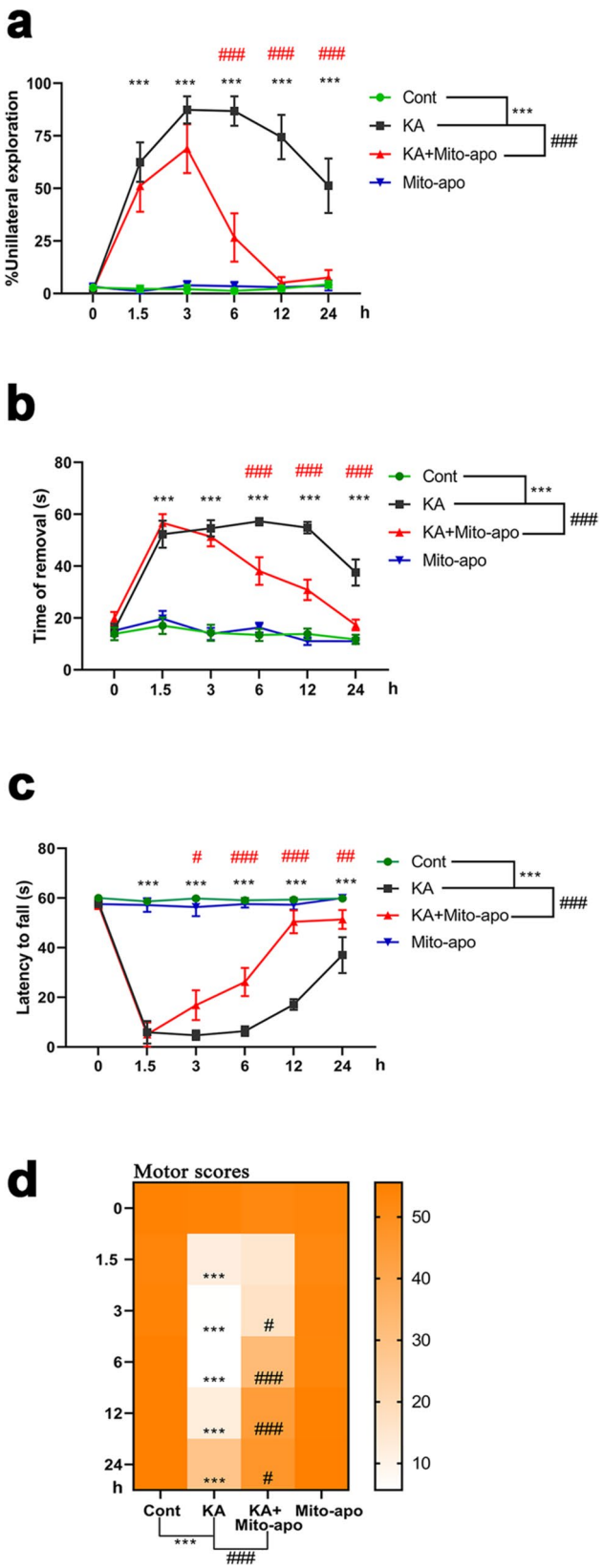

Fig. 5 Mito-apocynin reverses KA-induced motor behavioral impairment. Mice were administered 75 μg/kg Mito-apocynin 1 day in advance. Behavioral tests were performed at 0 h, 1.5 h, 3 h, 6 h, 12 h and 24 h after KA injection. (**a**) Cylinder test. (**b**) Adhesive removal test. (**c**) Inverted grid test. (**d**) Neurobehavioral dysfunction scores. $n=5$. Data are expressed as the mean ± SEM. *** $P<0.001$ versus control, # $P<0.05$ versus KA-treated group, ### $P<0.001$ versus KA-treated group, two-way ANOVA, Tukey test

of Mito-apocynin at 1 μM. Under conditions of excitotoxicity, mitochondrial damage results in insufficient ATP production and increased superoxide generation [43, 44], with the decrease in mitochondrial membrane potential being a critical step leading to cell death [9, 45]. Using an ATP detection kit, we observed that Mito-apocynin could improve the KA-induced reduction in ATP levels (Fig. 6c). JC-1 staining was employed to evaluate mitochondrial membrane potential; a decrease in this potential corresponds to a reduction in JC-1 aggregates (red fluorescence) within the mitochondrial matrix and an increase in JC-1 monomers (green fluorescence). Fluorescence observations demonstrated that Mito-apocynin could significantly reverse the KA-mediated decline in mitochondrial membrane potential (Fig. 6d-e). We measured mitochondrial superoxide levels using the MitoSOX Red mitochondrial superoxide indicator; an increase in red fluorescence intensity indicates elevated production of superoxides. The results revealed that KA increased mitochondrial superoxide levels, whereas Mito-apocynin significantly reversed the KA-mediated increase in mitochondrial superoxides (Fig. 6f-g). In summary, these findings suggest that Mito-apocynin treatment can alleviate KA-induced mitochondrial dysfunction *in vitro*.

Mito-Apocynin Inhibits KA-Induced Upregulation of Mitochondrial NOX4 and Promotes Restoration of the Mitochondrial Quality Control System

We hypothesize that mitochondrial NOX4 may play a pivotal role in mitochondrial damage associated with excitotoxicity. Mito-apocynin, a mitochondria-selective compound, effectively inhibits the KA-induced upregulation of mitochondrial NOX4 (Fig. 7a-c). The protective effect of Mito-apocynin appears to be contingent upon its ability to inhibit mitochondrial NOX4 expression, which subsequently reduces mitochondrial ROS production. Western blotting data indicate that Mito-apocynin treatment counteracts the KA-induced decrease in the phosphorylated DRP1 (Ser616)/total DRP1 ratio and significantly reverses the KA-induced upregulation of PGC-1α, PINK1, and Parkin (Fig. 7d-k). We conclude that the mitochondrion-targeted NOX inhibitor Mito-apocynin may mitigate KA-mediated mitochondrial damage by inhibiting alterations in mitochondrial NOX.

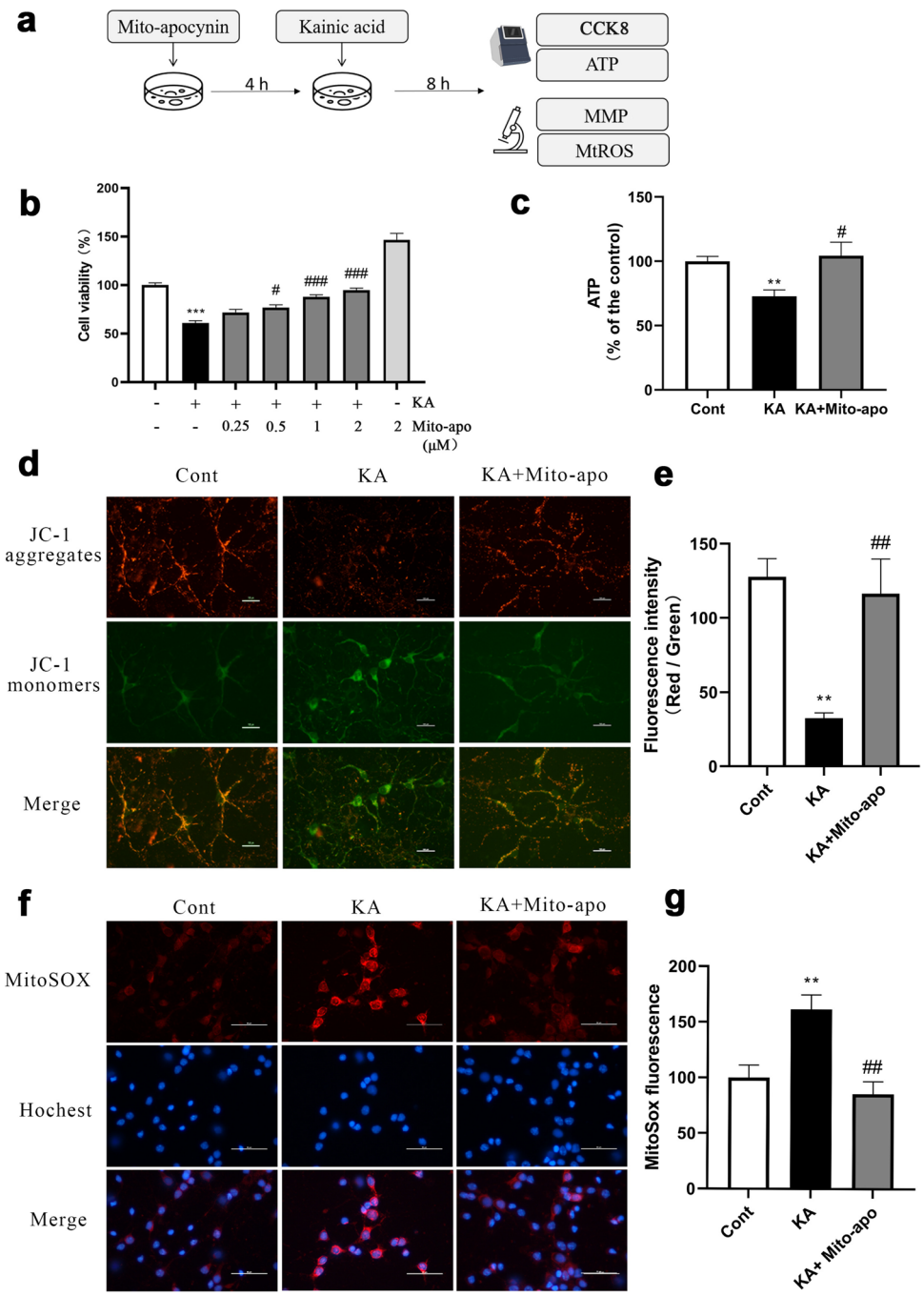

Fig. 6 Mito-apocynin reverses KA-induced primary neuronal damage and mitochondrial dysfunction *in vitro*. (**a**) Study road map of the experiment. Cells were pretreated with Mito-apocynin for 4 h and then treated with 100 μM KA for 8 h. (**b**) Cell viability of primary neurons. Cells were pretreated with Mito-apocynin (0.25, 0.5, 1 and 2 μM) for 4 h and then treated with KA for 8 h. Cell viability was determined by a CCK8 kit. $n=6$. (**c**) ATP levels were measured by an ATP detection kit. $n=6$. (**d**) Mitochondrial membrane potential was measured by a JC-1 detection kit. Scale bar = 100 μm. (**e**) Quantification of the fluorescence ratio (red fluorescence/green fluorescence). $n=6$. (**f**) Mitochondrial superoxide was measured by the MitoSOX Red probe. Scale bar = 50 μm. (**g**) Quantification of the MitoSOX fluorescence intensity. $n=6$. Data are expressed as the mean ± SEM. ** $P<0.01$ versus control, *** $P<0.001$ versus control, # $P<0.05$ versus KA-treated group, ## $P<0.01$ versus KA-treated group, ### $P<0.001$ versus KA-treated group, one-way ANOVA, Tukey test

Discussion

Glutamate is one of the most common neurotransmitters in the mammalian central nervous system, mediating excitatory neurotransmission [46]. However, high levels of glutamatergic input can cause excitotoxicity, leading to neuronal cell death after acute brain injury, such as stroke or trauma. In this study, we constructed an animal model of excitotoxicity by injecting the glutamate analog KA into the striatum. We found that KA-induced excitotoxicity impairs mitochondrial morphology and disrupts the quality control system. We also found that NOX appears to be involved in this process.

NOX is an enzyme that produces reactive ROS through the oxidation of NADPH. In the central nervous system, three primary NOX isoforms are expressed: NOX1, NOX2, and NOX4. In the KA-induced excitotoxicity model, we observed significant alterations in the expression of mitochondrial NOX4 and cytoplasmic NOX2, while the expression of NOX1 did not show any significant difference. The expression of NOX4 peaked at 6 h and subsequently

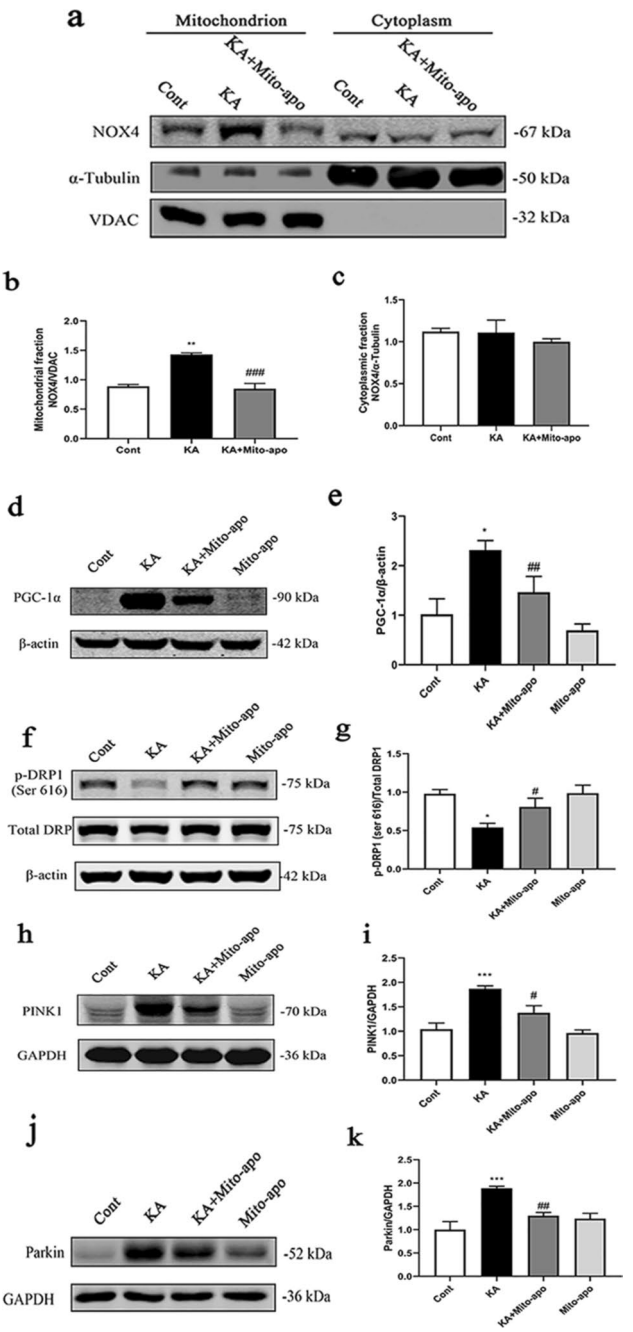

Fig. 7 Mito-apocynin reverses KA-induced upregulation of the expression levels of mitochondrial fraction NOX4 and ameliorates the disturbance of the mitochondrial quality control system. (**a**) Mice were pretreated with mito-apocynin (75 μg/kg) 1 day before KA (0.625 nmol) injection and then killed 6 h later, and striatal mitochondrial and cytoplasmic fractions were isolated. Representative image of a western blotting for NOX4. (**b**) Quantification of western blotting analysis of mitochondrial NOX4. $n=3$. (**c**) Quantification of western blotting for cytoplasmic NOX4. $n=3$. (**d**-**e**) Mice were pretreated with mito-apocynin (75 μg/kg) 1 day before KA (0.625 nmol) injection and then killed 48 h later to isolate proteins. Representative bands and semi-quantitation of western blotting for detecting PGC-1α. $n=4$. (**f**-**g**) Mice were pretreated with Mito-apocynin (75 μg/kg) 1 day before KA (0.625 nmol) injection and then killed 48 h later to isolate proteins. Representative bands and semi-quantitation of western blotting for detecting DRP1 (phospho-Ser616) and total DRP1. $n=5$. (**h**-**i**) Mice were pretreated with Mito-apocynin (75 μg/kg) 1 day before KA (0.625 nmol) injection and then killed 24 h later to isolate proteins for western blotting. Representative bands and semi-quantitation of western blotting for detecting PINK1. $n=4$. (**j**-**k**) Representative bands and semi-quantitation of western blotting for detecting Parkin. $n=6$. Data are expressed as the mean ± SEM. * $P<0.05$ versus control, ** $P<0.01$ versus control, *** $P<0.001$ versus control, # $P<0.05$ versus KA-treated group, ## $P<0.01$ versus KA-treated group, ### $P<0.001$ versus KA-treated group, one-way ANOVA, Tukey test

decreased gradually, which may be associated with its regulatory mechanisms within the cell.

In the early stages of KA-induced excitotoxicity, there is a sharp increase in intracellular oxidative stress. NOX4, a ROS-generating enzyme located in the mitochondria, may upregulate its expression as a rapid response mechanism to oxidative stress, thereby producing more ROS that participate in cell signaling and damage responses [47]. However, over time, cells may enhance their antioxidant enzyme systems, such as superoxide dismutase (SOD), to counteract the ROS generated by NOX4, resulting in a gradual decrease in NOX4 expression levels. Additionally, the interaction between NOX4 and the mitochondrial translocase TIM23 represents another regulatory mechanism [48]. TIM23 promotes mitochondrial ROS production and metabolic reprogramming of oxidative phosphorylation induced by NOX4, suggesting that this interaction may also influence changes in NOX4 expression.

In contrast, the expression of NOX2 peaks at 48 and 96 h, which may be attributed to its distinct regulatory mechanisms within the cell. NOX2 is predominantly located in the cell membrane, and its activity is modulated by various intracellular signaling pathways, including PI3K/Akt and MAPK [49, 50]. During KA-induced excitotoxicity, these signaling pathways may remain continuously activated [51], leading to the sustained high expression of NOX2. The ROS produced by NOX2 not only participate in extracellular signaling but may also exacerbate cellular damage by perpetuating the activation of intracellular signaling pathways [52].

The expression of NOX1, which showed no significant difference, may be attributed to its relatively minor role in excitotoxicity. NOX1 is primarily involved in diabetes and its complications. In both *in vivo* and *in vitro* models of diabetes-associated atherosclerosis in mice and humans, NOX1, rather than NOX4, has been identified as playing a major pathological role [53]. The expression of NOX1 is likely influenced predominantly by growth factors, cytokines, or other specific stress signals [54]. In the context of KA-induced excitotoxicity, intracellular oxidative stress and mitochondrial damage represent the primary

pathological mechanisms, leading to a relatively limited role for NOX1; consequently, its expression did not exhibit significant changes.

Studies have shown that the overactivation of glutamate receptors can lead to calcium overload, resulting in the disruption of mitochondrial morphology and subsequent mitochondrial dysfunction [55]. This underscores the importance of therapeutic measures targeting mitochondria. Given the distinct expression patterns of various NOX isoforms in the excitotoxicity model, we hypothesize that inhibiting the expression of mitochondrial NOX4 may rescue KA-induced mitochondrial damage. To investigate the potential role of NOX inhibitors in neuroprotection, we utilized Mito-apocynin, a mitochondria-targeted NOX inhibitor that exhibits greater mitochondrial selectivity compared to other NOX inhibitors. Mito-apocynin can effectively reach the striatum when administered orally. Our study demonstrates that supplementation with Mito-apocynin can mitigate excitotoxic damage. However, it is important to note that apocynin is a nonspecific NOX inhibitor. Although Mito-apocynin successfully inhibited the KA-induced upregulation of mitochondrial NOX4, it remains challenging to rule out its effects on other NOX isoforms present in mitochondria. Furthermore, the absence of suitable animal models to elucidate the subtype- and subcellular-specific functions of NOX4 *in vivo* necessitates further in-depth studies to clarify the role of NOX4 in excitotoxicity-mediated mitochondrial injury and neurodegenerative diseases.

In summary, during KA-induced excitotoxicity, the upregulation of mitochondrial NOX expression coincides with mitochondrial dysfunction and impairment of the mitochondrial quality control system. Mito-apocynin, as an exogenous mitochondrion-targeted NOX inhibitor, can exert neuroprotective effects by inhibiting mitochondrial NOX expression and ameliorating mitochondrial dysfunction and quality control system impairment, thereby reversing KA-induced mitochondrial damage (Fig. 8).

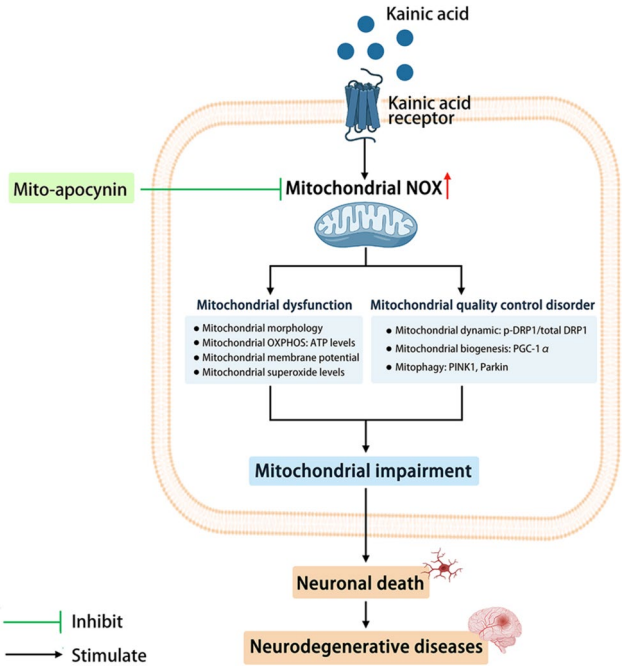

Fig. 8 Mito-apocynin protects against KA-induced excitotoxicity by attenuating mitochondrial impairment. KA upregulated the expression of the mitochondrial fraction NOX protein. KA led to mitochondrial dysfunction and disrupted the mitochondrial quality control system. Exogenous supplemental mitochondrion-targeted NOX inhibitor Mito-apocynin ameliorated KA-induced mitochondrial impairment by inhibiting mitochondrial NOX upregulation

Declarations

Ethics Approval The study has been examined and certified by the Ethics Committee of Soochow University, and informed consent was obtained from all participants included in the study, in agreement with institutional guidelines.

Consent for Publication All authors have approved for publication.

Competing Interests The authors declare no competing interests.

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Author Contribution M.L. and Y.W. designed the study. H.W., X.W., N.L., Y.J., Y.S., and J.W. assisted with the experiments. M.L., Y.W., H.X., J.X., and Z.Q. contributed to the drafting of the manuscript and figures. All authors have approved the final article.

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Data Availability No datasets were generated or analysed during the current study.

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