Experimental Neurology 320 (2019) 112988

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research paper

Blockade of voltage-gated potassium channels ameliorates diabetes-associated cognitive dysfunction in vivo and in vitro

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ARTICLE INFO

Keywords:
Diabetes-associated cognitive dysfunction
Tetraethylammonium
4-Aminopyridine
Hippocampus
Oxidative stress
Apoptosis

ABSTRACT

The voltage-gated potassium (Kv) channel blockers tetraethylammonium (TEA) and 4-aminopyridine (4-AP) have shown beneficial effects on some neurological disorders. But their involvements in diabetes-associated cognitive dysfunction are still unknown. The present study aims to investigate whether the blockade of Kv channels by TEA and 4-AP alleviate cognitive decline in diabetes. In vivo, the effects of TEA and 4-AP (5 mg/kg body weight per day, 1 mg/kg body weight per day intraperitoneal injected for 4 weeks, respectively) were investigated in streptozotocin-induced C57BL/6 diabetic mice. In vitro study, we investigated the effects of TEA and 4-AP on the high glucose (HG) -stimulated primary cortical neurons. The results showed that TEA and 4-AP ameliorated the cognitive decline of diabetic mice in the Morris water maze test, improved the ultrastructure of pancreatic β cells, hippocampal neurons and synapses, decreased oxidative stress, modulated apoptosis-related proteins, and activated phosphatidylinositol 3-kinase (PI3K)/ Protein kinase-B (PKB or Akt) signaling pathway. In the HG-stimulated primary cultured cortical neurons, TEA and 4-AP increased the cell viability, decreased oxidative stress; prevented apoptosis and activated PI3K/Akt signaling pathway. Additionally, the PI3K inhibitor LY294002 partially abolished the effects of TEA and 4-AP. These findings indicate that the blockade of Kv channels by TEA and 4-AP ameliorates the diabetes-associated cognitive dysfunction via PI3K/Akt pathway, suggesting that targeting Kv channels could be a promising strategy for the treatments of cognitive impairments in diabetes.

1. Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia, and affects individuals worldwide. It is mainly caused by defects in insulin production or sensitivity. Long-term chronic hyperglycemia can damage the central nervous system (CNS). DM leads to a wide range of changes in the neural structure of the brain (Zhang et al., 2017b; Zhao et al., 2016; Zhou et al., 2016a), neurotransmitter levels (Han et al., 2018; Markowicz-Piasecka et al., 2017; Shpakov et al., 2015), electrophysiology (He et al., 2016; Heng et al., 2011; Yang et al., 2016), and blood circulation (Iwase et al., 2017; Sorensen et al., 2016). Diabetes-associated cognitive decline is a clinical complication of DM that manifests as a deficit in reasoning ability, learning and memory deficits, and a lack of attention, among other symptoms. The mechanisms underlying the cognitive impairments in patients with diabetes have not been clearly identified. Nevertheless, the pathogenesis is linked to alterations in the brain structure, electrophysiological deficits, oxidative injury, and neuronal apoptosis (Gaspar et al., 2016).

Voltage-gated potassium (Kv) channels are widely expressed in the CNS and are involved in the generation and transmission of neuronal excitability, neurotransmitter release, cell proliferation, degradation and death. According to the pharmacological and physiological function and electrophysiological properties, Kv channels mainly consist of delayed rectifier potassium channels and transient outward potassium channels (or A-type channels). Delayed rectifier potassium channels are related to the neuronal apoptosis while A-type channels play an

Abbreviations: Kv, voltage-gated potassium; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; PI3K, phosphatidylinositol 3-kinase; Akt, Protein kinase-B; HG, high glucose; DM, diabetes mellitus; AD, Alzheimer's disease; PD, Parkinson's disease; Aβ, amyloid β protein; TEM, transmission electron microscope; MTT, methylthiazolyldiphenyl tetrazolium; SOD, superoxide dismutase; MDA, malondialdehyde; FBG, fasting blood glucose; LY, LY294002

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https://doi.org/10.1016/j.expneurol.2019.112988
Received 21 September 2018; Received in revised form 29 March 2019; Accepted 25 June 2019
Available online 26 June 2019
0014-4886/

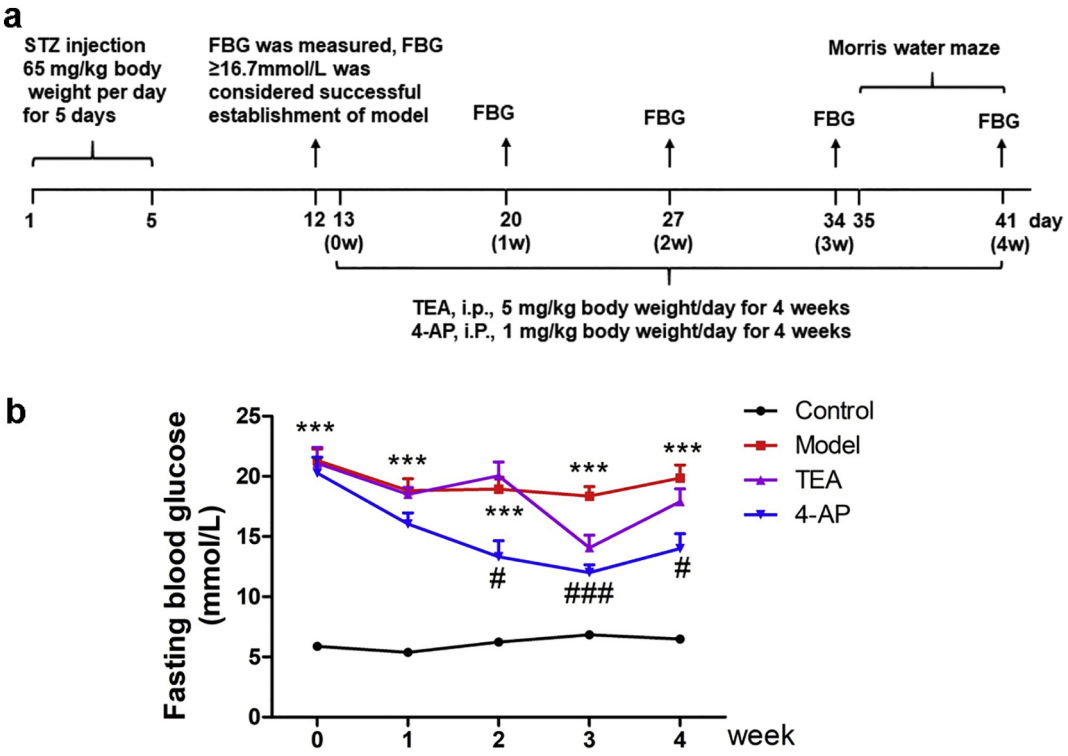

Fig. 1. (a) The experimental design of in vivo study. C57BL/6 J mice were intraperitoneally injected with STZ (65 mg/kg body weight, lasted for 5 days) to induce T1DM model. In the 7th day after STZ administration, fasting blood glucose was measured by snipping the tails. If the fasting blood glucose was higher than 16.7 mmol/L, the diabetes model was considered successfully induced. Then diabetic mice were randomly divided into Model, TEA (5 mg/kg body weight), and 4-AP (1 mg/kg body weight) group. The drug treatment lasted for 4 weeks. FBG levels were measured weekly during 4 weeks of TEA and 4-AP treatment. At the 4th week following treatment, the Morris water maze test was used to evaluate the cognitive performance of C57BL/6 J mice. Then the mice were sacrificed to isolate hippocampus and pancreas for further experiments. (b) Effects of TEA and 4-AP on the FBG levels of STZ-induced diabetic mice. Data are expressed as mean ± SEM. $^{***}P < .001$, versus the control group; $^{\#}P < .05$, $^{\#\#\#}P < .001$, versus the model group. $n = 10$.

important role in hippocampal synaptic plasticity. Some Kv channels are abnormally expressed in several neurodegenerative disease such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Boscia et al., 2017; Rangaraju et al., 2015; Subramaniam et al., 2014; Yin et al., 2017). Substance P (SP), a neuropeptide broadly distributed in the CNS, has been demonstrated to recover the beta amyloid-induced cognitive impairments in rats (Campolongo et al., 2013). This protective effect could be due to SP regulation of Kv channel subunits expression, thus inhibiting the efflux of K^+ (Pieri et al., 2010; Amadoro et al., 2007), which is essential for controlling neuronal apoptosis. Many researchers have focused on the effects of Kv channels on insulin secretion in subjects with diabetes (Fu et al., 2017; Henquin et al., 2017). In addition, Heng et al. reported an enhancement of voltage-gated potassium currents in the CA1 pyramidal neurons of streptozotocin (STZ)-induced diabetic rats with impaired learning performance (Heng et al., 2011).

Potassium channel blockers have protective effect on neurodegeneration. Tetraethylammonium (TEA), a non-selective delayed rectifier potassium channel blocker, decreases the neuronal apoptosis induced by amyloid β protein (Aβ) (Wang et al., 2012; Yu et al., 2006). 4-Aminopyridine (4-AP), which nonspecifically blocks A-type channels, exerts beneficial therapeutic effects on some neurological disorders, such as ataxia and multiple sclerosis (Jayabal et al., 2016; Keune et al., 2015). Both TEA and 4-AP exert positive effects on PD symptoms in an animal model of 6-OHDA-induced PD (Haghdoost-Yazdi et al., 2011; Haghdoost-Yazdi et al., 2017). However, their roles in the diabetes-associated cognitive dysfunction have been rarely elucidated. We hypothesized that the Kv channel blockers TEA and 4-AP inhibit the DM-induced alterations in the activity of Kv channels, affecting neuronal survival and synaptic plasticity, and subsequently influencing learning and memory process. To test the hypothesis, we studied the effects of TEA and 4-AP on the STZ-induced diabetic mice with cognitive dysfunction and high glucose (HG) -stimulated rat primary cortical neurons, and identify the underlying mechanisms.

2. Materials and methods

2.1. Induction of diabetes model and drug administration

All animal experimental protocols were approved by the Institutional Animals Care and Use Committee at Xi'an Jiaotong University and in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The male C57BL/6 J mice weighing 18–22 g were purchased from Medical Experimental Animal Center of Xi'an Jiaotong University. The mice were maintained in a temperature-controlled environment with a constant 12-h light/dark cycle and ad libitum access to water. After fasted for 6 h, 65 mg/kg body weight STZ (Sigma-Aldrich, USA) was intraperitoneally injected for consecutive 5 days to induce diabetes. Control C57BL/6 J mice ($n = 10$) were injected with equivalent volume of citrate buffer. In the 7th day after STZ administration, fasting blood glucose was measured by snipping the tails. If the fasting blood glucose was higher than 16.7 mmol/L, the diabetes model was considered successfully induced. The success rate was over than 85%. Then the model mice were randomly divided into 3 groups (n = 10 per group): (1) Model group, in which the mice were intraperitoneally injected with normal saline. (2) TEA group, in which the mice were intraperitoneally injected with TEA (Sigma-Aldrich, USA) at a dose of 5 mg/kg body weight for 4 weeks; (3) 4-AP group, in which the group were intraperitoneally injected with 4-AP (Sigma-Aldrich, USA) at a dose of 1 mg/kg body weight for 4 weeks. The doses of TEA and 4-AP were determined by previous references

(Haghdoost-Yazdi et al., 2011; Haghdoost-Yazdi et al., 2016; Haghdoost-Yazdi et al., 2017). The fasting blood glucose was monitored every week. At the 4th week during the drug administration, Morris water maze test was conducted. When the test was finished, all the mice were sacrificed by cervical dislocation to isolate the pancreas and hippocampus tissues. The summary of experimental design was shown in Fig. 1a.

2.2. Morris water maze

Morris water maze was used to evaluate the cognitive function of diabetic mice. The test consisted of acquisition trial and probe trial. The acquisition trial lasted for 5 days, 4 times a day. A hidden platform was located in 2 cm below the water surface of circular pool which was divided into four identical quadrants. During the experiment, each mouse was force to finish a swim test to find the hidden platform within 90 s. The formal test began at 2nd day. In the 1st day, each mouse was allowed to swim freely in the pool to adapt for the environment. The time each mouse spent to reach the platform for the first time (escape latency) and the swimming distance in this period were recorded. The probe trail was conducted on 6th day. The hidden platform was removed and each mouse freely swam in the pool within 60 s. The number of times across the platform and the time spent in effective area were recorded.

2.3. Transmission electron microscope (TEM)

The hippocampus and pancreas were removed and cut into 1mm^3 cubes, fixed with 2.5% glutaraldehyde and 2% osmic acid, then dehydrated and embedded in epoxy resin. Ultrathin sections were collected onto 200-mesh copper grids, double stained with uranyl acetate and lead acetate, and then observed under Hitachi H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).

2.4. Primary cortical neuron cultures

The method of primary cell culture was conducted as previously described (Zhang et al., 2017a). Primary cultures of rat cortical neurons were prepared from E18 to E19 Sprague-Dawley rat embryos. The pregnant SD rat was anesthetized with 10% chloral hydrate at a dose of 3 mL/kg body weight. Then the embryos were removed to isolate the brains, 6 to 8 embryos were used based on our experimental arrangements. The cerebral cortices were dissected from the brain in sterile ice-cold PBS with the blood vessels and meninges carefully removed. Then isolated cerebral cortices were cut into small blocks and pipetted 20 times in 5 mL DMEM medium (Hyclone, USA). Undispersed pieces were filtered through a nylon mesh with a pore size of 75 μm, and then the supernatant was transferred to a new sterile 15 mL tube and centrifuged at 200*g* for 3 min. Cell aliquots were resuspended, and viable cells were counted using a trypan blue dye exclusion assay. The cells were then dissociated in Neurobasal medium (Gibco, USA) supplemented with 2% B27 supplement (Gibco, USA), 0.25% GlutaMax (Gibco, USA), 0.5% penicillin/streptomycin (Solarbio, China). The cortical neurons were plated on poly-L-ornithine hydrobromide-coated 96- well plate (5×10^4 cells/well) or 6- well plate (2×10^6 cells/well). The cultures were maintained in a humidified incubator with 5% CO_2/95% air at 37 °C. Half of the medium was replaced by fresh medium every three days.

2.5. Cell viability assay

The cell viability was measured by methylthiazolyldiphenyl tetrazolium (MTT) assay. The cortical neurons were seeded in poly-L-ornithine hydrobromide-coated 96 well plate at a density of 5 × 104 per well. After cultured for 7 days, the cortical neurons were exposed to different concentrations of glucose (0, 25, 50, 75, 100 mmol/L) and potassium channel blockers TEA (1, 2.5, 5.0 mmol/L) and 4-AP (0.25, 0.5, 1.0, 2.0 mmol/L). And the neurons were cultured for another 48 h. Then 20 μL MTT (Sigma-Aldrich, USA) was add into each well and incubated for 4 h. Then remove the cultured medium and add 150 μL DMSO into each well to dissolve the formazan dye. The absorbance at 490 nm was measured by ELISA reader (BioTek, USA). Cell viability was calculated by the ratio of OD value of each group to that of control group.

2.6. Biochemical analysis

The hippocampus tissues isolated from C57BL/6 J mice and primary cultured cortical neurons were used for Biochemical analysis. The superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels were measured using a total SOD assay kit (hydroxylamine method) and a MDA assay kit (TBA method) according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). 1 unit of SOD activity was defined as the amount of SOD per milligram of protein in the 1 mL solution when the inhibition rate of SOD was 50%. MDA levels were expressed as nanomoles of MDA per milligram of protein.

2.7. Western blot analysis

Western blot analysis was performed as previously described (Wang et al., 2017). The hippocampus tissues and primary cortical neurons were homogenized in ice-cold RIPA buffer containing the Halt protease inhibitor cocktail (Roche, Basel, Switzerland). Lysates were clarified by centrifugation at 12,000*g* for 10 min at 4 °C. Then the protein concentration of each sample was determined by using a BCA protein assay kit (Heart, China). 30 to 50 micrograms of proteins from each sample were separated by 10%~15% SDS-PAGE gel electrophoresis and then transferred to PVDF membranes (Millipore, USA). After blocked in 5% (*w/v*) non-fat milk for 2 h, the membranes were incubated with primary antibodies against Kv4.2 (Cell Signaling Technology, USA), Kv2.1 (Sigma-Aldrich, USA), cleaved caspase 3 (Cell Signaling Technology, USA), Bcl-2 (Sigma-Aldrich, USA), Bax (Cell Signaling Technology, USA), PI3K (Abcam, UK), p-PI3K (Tyr458) (Cell Signaling Technology, USA), phospho-Akt2 (Ser474) (Cell Signaling Technology, USA; Abcam, UK), Akt2 (Epitomics, USA), GSK-3β (Cell Signaling Technology, USA) and p-GSK-3β (Ser9) (Cell Signaling Technology, USA) at 4 °C overnight. After washed thrice with TBST, the membranes were incubated with corresponding HRP-conjugated secondary antibodies at room temperature for 2 h. The membranes were washed thrice with TBST and then visualized by using ECL substrate solution (Genshare, China).

2.8. Statistical analysis

Data were analyzed by IBM SPSS 20.0 software. All results were expressed as mean ± SEM. Multiple comparisons were analyzed by one-way ANOVA followed by Fisher's least significant difference test ((LSD-t, if the data were normally distributed) or Dunnett T3 (if the data were not normally distributed). For Morris water maze test, the data were analyzed by using a multivariate analysis of variance (MANOVA) of repeated measures for comparisons among trials. For western blot, the band intensity was quantified by using ImageJ. All *P* values were two-sided, and a value of $P < .05$ was considered statistically significant. The pictures were processed by GraphPad Prism 5.

3. Results

3.1. Effects of TEA and 4-AP on the fasting blood glucose levels of STZ-induced diabetic mice

A summary of the experimental design is shown in Fig. 1a. The STZ injection resulted in a diabetic syndrome that was verified by the

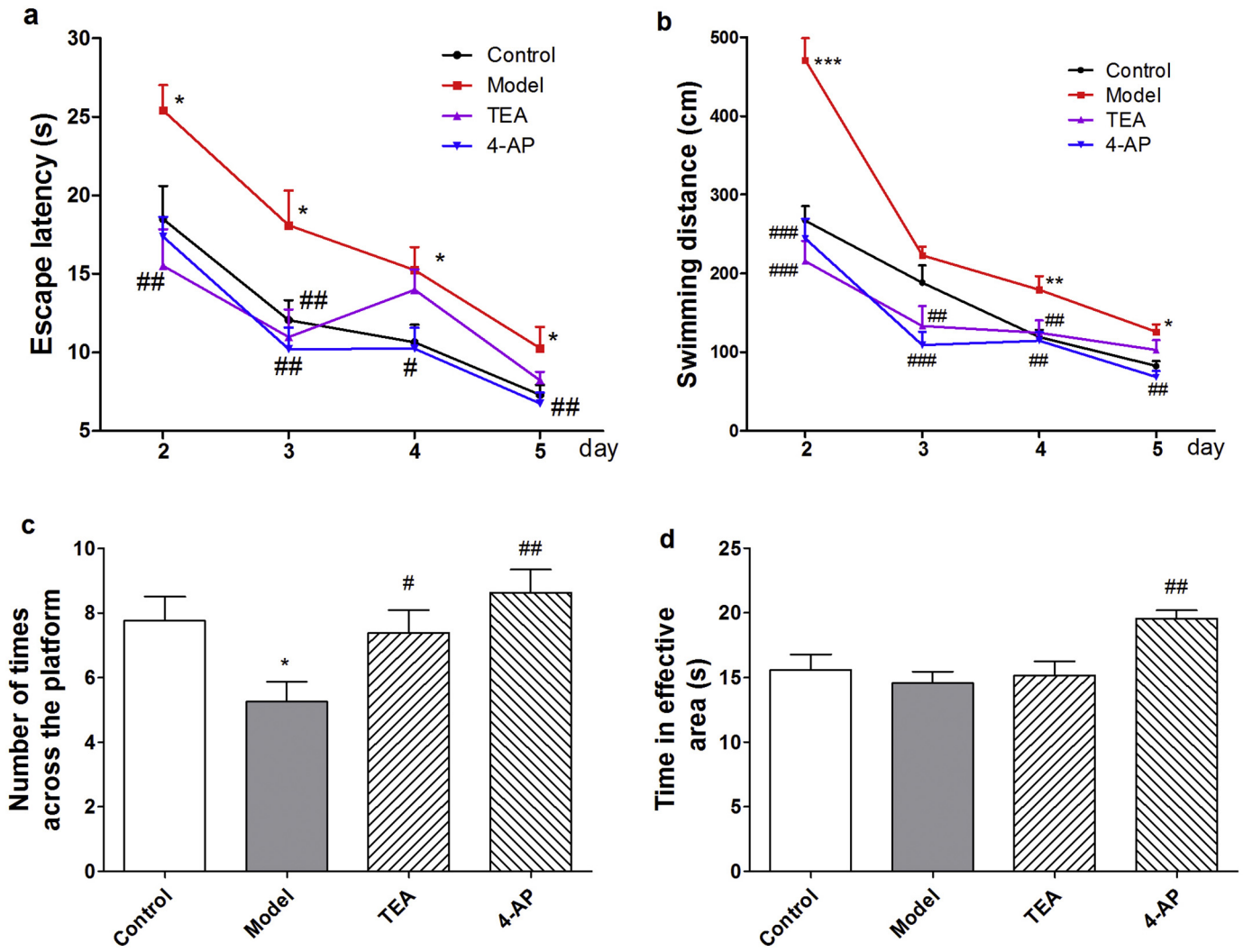

Fig. 2. The cognitive performance of STZ-induced diabetic mice in Morris water maze. (a) The escape latency and (b) swimming distance during the acquisition trials with a hidden platform. (c) The number of times across the platform and (d) time in effective area during the probe trial. Data are expressed as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, versus the control group; $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$, versus the model group. $n = 8$.

presence of polydypsia, polyuria, and hyperglycemia in the diabetic animals. After successfully establishing the diabetes model, weekly changes in fasting blood glucose (FBG) levels in the control, model, TEA and 4-AP groups were determined with a blood glucometer on the last day of every week, and measured from the tail veins. As shown in Fig. 1b, significantly higher baseline FBG levels were observed in the model group than in the control group. A significant difference in FBG levels was not observed between the TEA-treated group and the model group. However, 4-AP-treated group exhibited significantly reduced FBG levels beginning at the 2nd week compared with the model group.

3.2. The potassium channel blockers TEA and 4-AP ameliorate the learning and memory impairments in STZ-induced diabetic mice

The Morris water maze test was used to evaluate the cognitive performance of diabetic mice. As illustrated in Fig. 2, the diabetic mice exhibited significant learning and memory deficits in the acquisition trials and probe trial. Specifically, the diabetic mice showed a significantly increased escape latency (Fig. 2a, $P < .05$) and increased swimming distance (Fig. 2b, $P < .05$) in the acquisition trials compared with the control group. In the probe trial, the diabetic mice crossed the location of the platform significantly fewer times (Fig. 2c, $P < .05$) and spent less time in the effective area (Fig. 2d). The treatment groups required less time and distance to locate the hidden platform in the acquisition trials. In the probe trials, the treatment groups crossed the platform more frequently and spent more time in the effective area than the model group. Thus, TEA and 4-AP ameliorate the learning and memory impairments in STZ-induced diabetic mice. Additionally, the 4-AP treatment exerts better effects on diabetic mice than TEA.

3.3. Effects of TEA and 4-AP on the ultrastructure of pancreatic β cells in STZ-induced diabetic mice

Pancreatic β cells, which are located in the pancreatic islets, are secretory cells that secrete insulin. They are essential for maintaining glucose homeostasis. When the functions of pancreatic β cells are impaired, insulin secretion will be absolutely or relatively inadequate, thus resulting in an elevated blood glucose level. The ultrastructure of pancreatic β cells was observed under TEM. As shown in Fig. 3, normal β cells contain abundant secretory granules with an electron-dense core and clear halo. Compared with the control group, the β cells in the diabetic mice exhibited fewer insulin secretory granules and more empty vesicles. Swollen mitochondria and destroyed mitochondrial cristae were also observed in the model group. Consistent with the observation that TEA did not decrease the FBG level in the diabetic

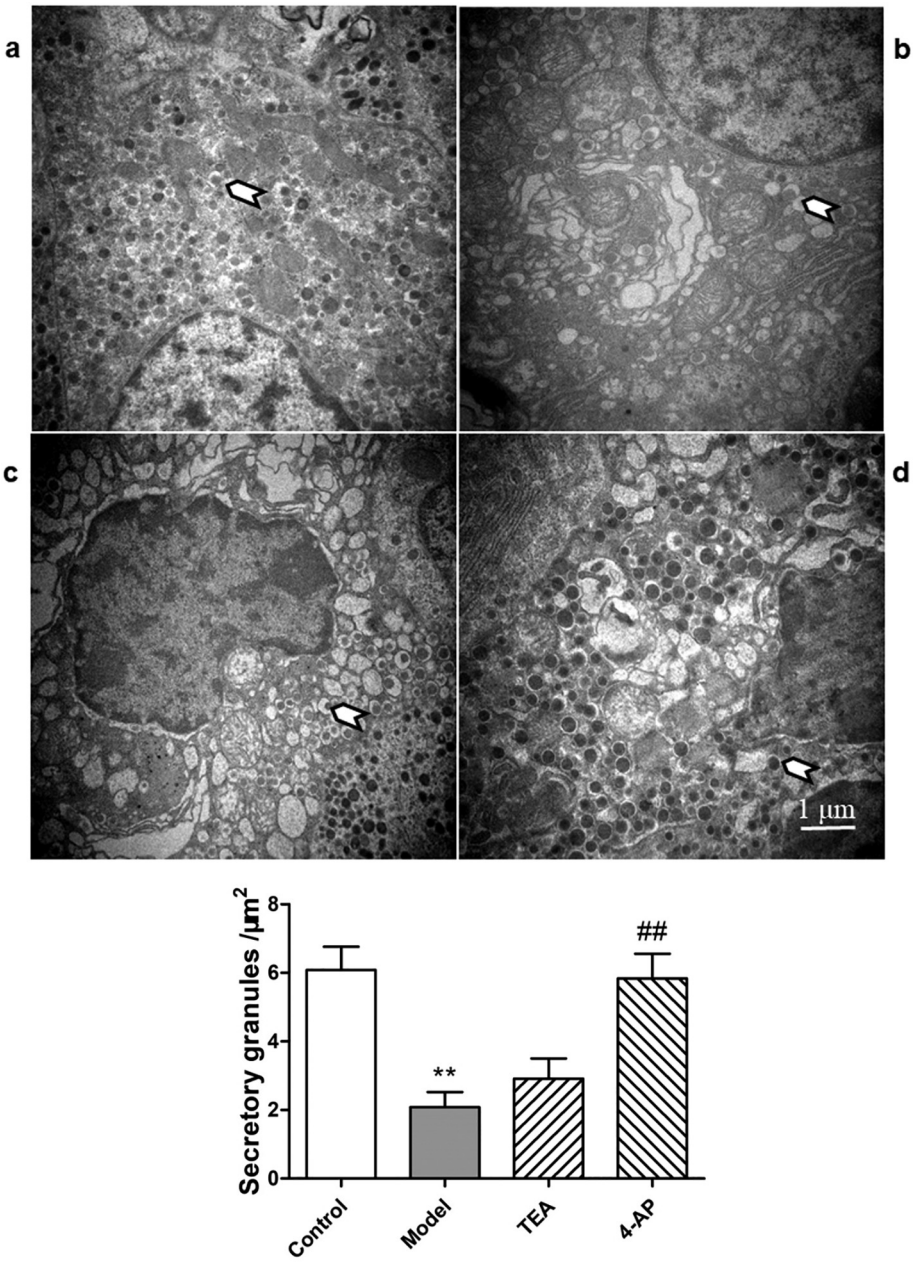

Fig. 3. Effects of potassium channel blockers TEA and 4-AP on the ultrastructure of pancreatic β cells. The ultrastructure of pancreatic β cells was observed under TEM (20,000 ×). (a-d) showed the ultrastructure of pancreatic β cells in the control, model, TEA, and 4-AP group, respectively. The white arrows represent the insulin secretory granules. Scale bar, 1 μm. (e) The insulin secretory granules in each group. Data are expressed as mean ± SEM. $^{**}P < .01$, versus the control group; $^{\#\#}P < .01$, versus the model group. $n = 3$.

mice, a significant difference in the number of insulin secretory granules was not observed between the TEA group and the model group. Compared with the model group, the 4-AP treatment significantly increased the number of insulin secretory granules (Fig. 3e, $P < .01$) and decreased the number of empty vesicles.

3.4. TEA and 4-AP improve the ultrastructure of hippocampal neurons and synapses in STZ-induced diabetic mice

The hippocampus, a key brain region responsible for learning and memory, is vulnerable to changes in glucose homeostasis. The ultrastructure of hippocampal neurons in STZ-induced diabetic mice was observed under TEM. The diabetic mice exhibited decreased numbers of neurons, obscure boundaries and abnormally distributed chromatin compared with the control mice (Fig. 4a). However, the TEA and 4-AP treatments reduced the injuries. Compared with the model group, the numbers of hippocampal neurons were increased, the nuclear membrane was smoother and the chromatin was normally distributed in the TEA and 4-AP groups. Neuronal synapses were also observed (Fig. 4b). The model group showed decreased numbers of synapses and the postsynaptic density was thinner than the control group. Compared with the model group, treatments with TEA and 4-AP increased the number of synapses and improved the synaptic structure.

3.5. TEA and 4-AP attenuate oxidative stress in the hippocampus of STZ-induced diabetic mice

Oxidative stress is involved in diabetes-associated cognitive dysfunction. Compared with the control group, the hippocampus of STZ-induced diabetic mice exhibited significantly decreased SOD activities

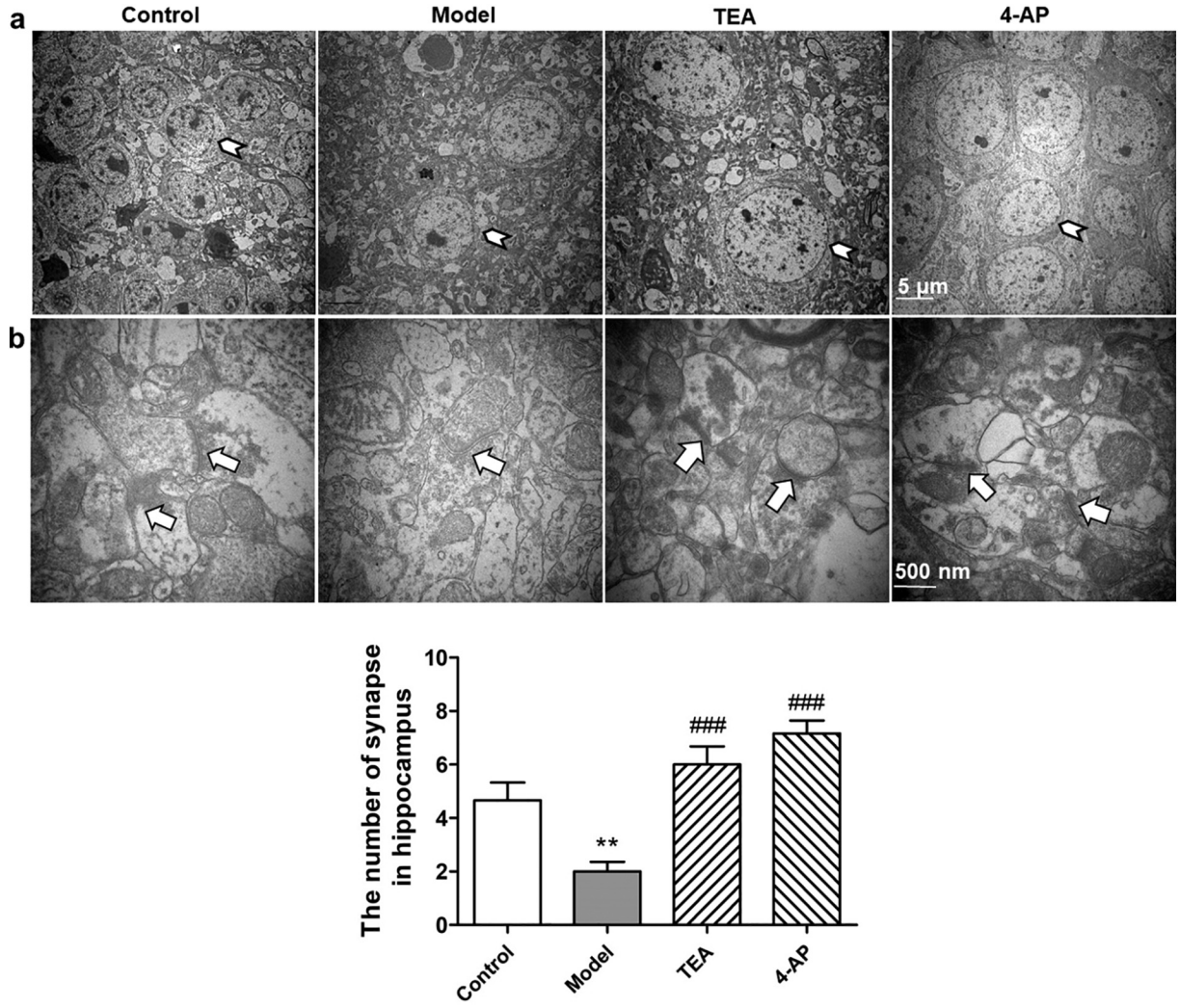

Fig. 4. TEA and 4-AP improve the ultrastructure of hippocampal neurons and synapses in STZ-induced diabetic mice. The ultrastructure of hippocampus neurons and synapses was observed under TEM. (a) The representative pictures of hippocampus neurons (4000 ×) from each group. The arrowheads represent the hippocampal neurons. Scale bar, 5 μm. (b) The representative picture of neuronal synapses (40,000 ×) from each group. The white arrows represent the hippocampal synapses. Scale bar, 500 nm. (c) The number of synapses in hippocampus. The hippocampus tissue for TEM was obtained from 3 mice in each group. The data were analyzed in 6 randomly chosen images per group. Data are expressed as mean ± SEM. $^{**}P < .01$, versus the control group; $^{###}P < .001$, versus the model group.

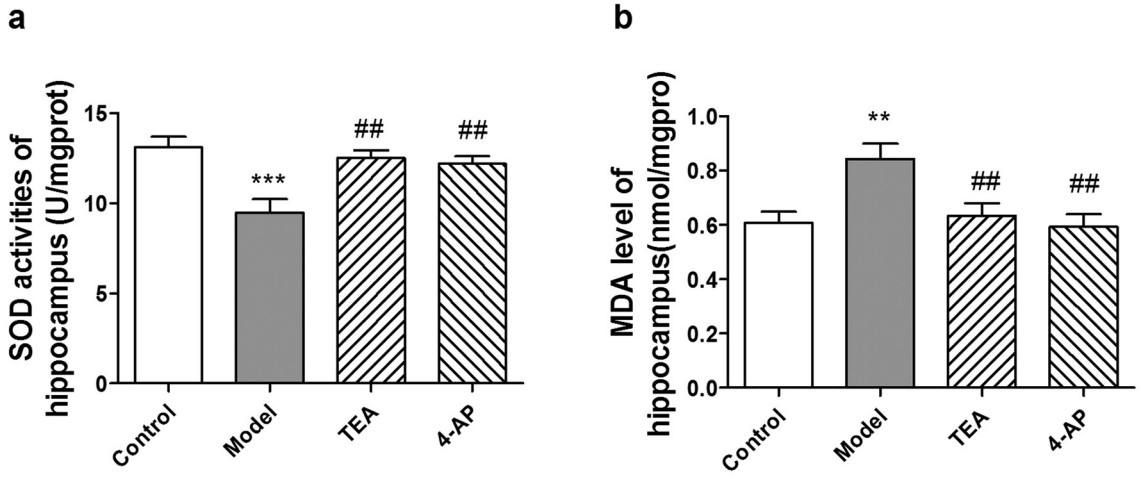

Fig. 5. Effects of TEA and 4-AP on the SOD activities (a) and MDA levels (b) in the hippocampus of STZ-induced diabetic mice. $^{*}P < .05$, versus the control group; $^{###}P < .001$, versus the model group. $n = 5$.

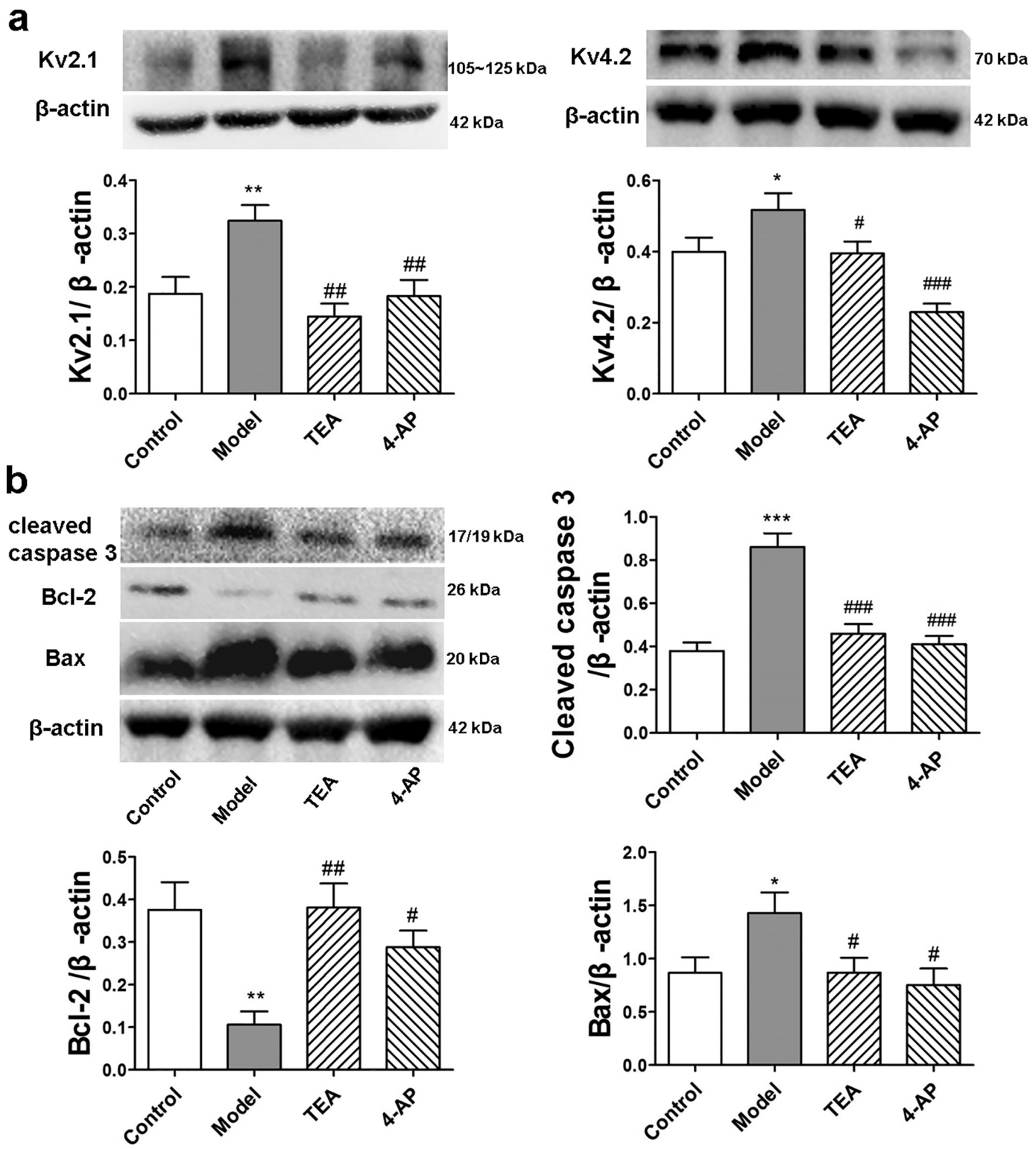

Fig. 6. Kv channels blockade prevents apoptosis in the hippocampus of STZ-induced diabetic mice. (a) Represent proteins bands and quantification of Kv channel subunits Kv2.1 and Kv4.2. For Kv2.1, $n = 4$; for Kv4.2, $n = 5$. (b) Represent proteins bands and quantification of apoptosis-related proteins cleaved caspase 3, Bcl-2 and Bax. For cleaved caspase 3 and Bax, n = 4; for Bcl-2, n = 5. The data are expressed as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, versus the control group; $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$, versus the model group.

(Fig. 5a, $P < .001$) and increased MDA levels (Fig. 5b, $P < .01$). In contrast, treatments with TEA and 4-AP significantly increased SOD activities and decreased MDA levels compared to the model group.

3.6. Kv channel blockade prevents apoptosis in the hippocampus of STZ-induced diabetic mice

Kv2.1, a predominant delayed rectifier potassium channel in hippocampal, cortical, and granule neurons, plays an important role in the

neuronal apoptosis (Liu et al., 2018; Zhou et al., 2016b). Kv4.2 is a major A-type channel in the central nervous system. Alterations in the function and activities of Kv4.2 influence the components of *N*-methyl-D-aspartic acid (NMDA) receptor subunits (Jung et al., 2008; Kaufmann et al., 2013), thus affecting synaptic plasticity, learning and memory. The protein levels of Kv2.1 and Kv4.2 in hippocampus of STZ-induced diabetic mice were detected using Western blotting. As shown in Fig. 6a, the model group exhibited a significant upregulation of Kv2.1 and Kv4.2 compared with the control group. Compared with the model group, TEA and 4-AP both inhibited the upregulation of Kv2.1 and Kv4.2.

We also detected the expression of apoptosis-related proteins, such as cleaved caspase 3, Bcl-2 and Bax (Fig. 6b). Compared with the control group, the model group showed significantly increased expression of cleaved caspase 3 and Bax, and decreased expression of Bcl-2. Compared with the model group, TEA and 4-AP decreased expression of cleaved caspase 3 and Bax, and increased expression of Bcl-2.

3.7. TEA and 4-AP activate the PI3K/Akt signaling pathway in the hippocampus of STZ-induced diabetic mice

As the PI3K/Akt signaling pathway regulates apoptosis and survival, we tested whether TEA and 4-AP modulate the effects of the PI3K/Akt signaling pathway on the development of diabetes-associated cognitive dysfunction. As shown in Fig. 7, compared with the control group, the expression of PI3K, phosphorylated PI3K (p-PI3K), Akt2 and p-Akt2 were significantly decreased, and the expression of GSK-3β and p-GSK-3β were markedly increased in the model group. However, TEA and 4-AP upregulated the expression of PI3K , p-PI3K, Akt2 and p-Akt2, and downregulated the expression of GSK-3β and p-GSK-3β.

3.8. TEA and 4-AP inhibit high glucose (HG)-induced damage of primary cortical neuron

Primary cortical neurons were exposed to increasing concentrations of glucose (0, 25, 50, 75, or 100 mmol/L) for 48 h to determine the concentration of glucose that induced neuronal death, with mannitol serving as the isotonic control. Cell viability was then measured using a modified MTT assay. As shown in Fig. 8a, glucose decreased the viability of primary cortical neurons in a dose-dependent manner. A 50 mmol/L glucose treatment was chosen as the model condition.

Initially, we adopted gradient concentration of TEA (1, 2.5, or 5.0 mmol/L) and 4-AP (0.25, 0.5, 1.0, or 2.0 mmol/L) to investigate their effects on HG-stimulated primary cortical neurons. As illustrated in Fig. 8b and c, TEA and 4-AP protected primary cortical neurons from HG-induced cell death. However, their protective effects were not dose-dependent. We chose 2.5 mmol/L TEA and 0.5 mmol/L 4-AP for use in subsequent experiments because these concentrations exerted the maximum effect.

3.9. TEA and 4-AP ameliorate the oxidative stress induced by HG in primary cortical neurons

In the present study, 50 mmol/L glucose induced oxidative stress in cultured primary cortical neurons. This result demonstrated that HG significantly decreased the SOD activity (Fig. 9a, $P < .05$) and increased the MDA levels (Fig. 9b, $P < .001$) in primary cortical neurons compared with the control group. As shown in Figs. 9, 2.5 mM TEA and

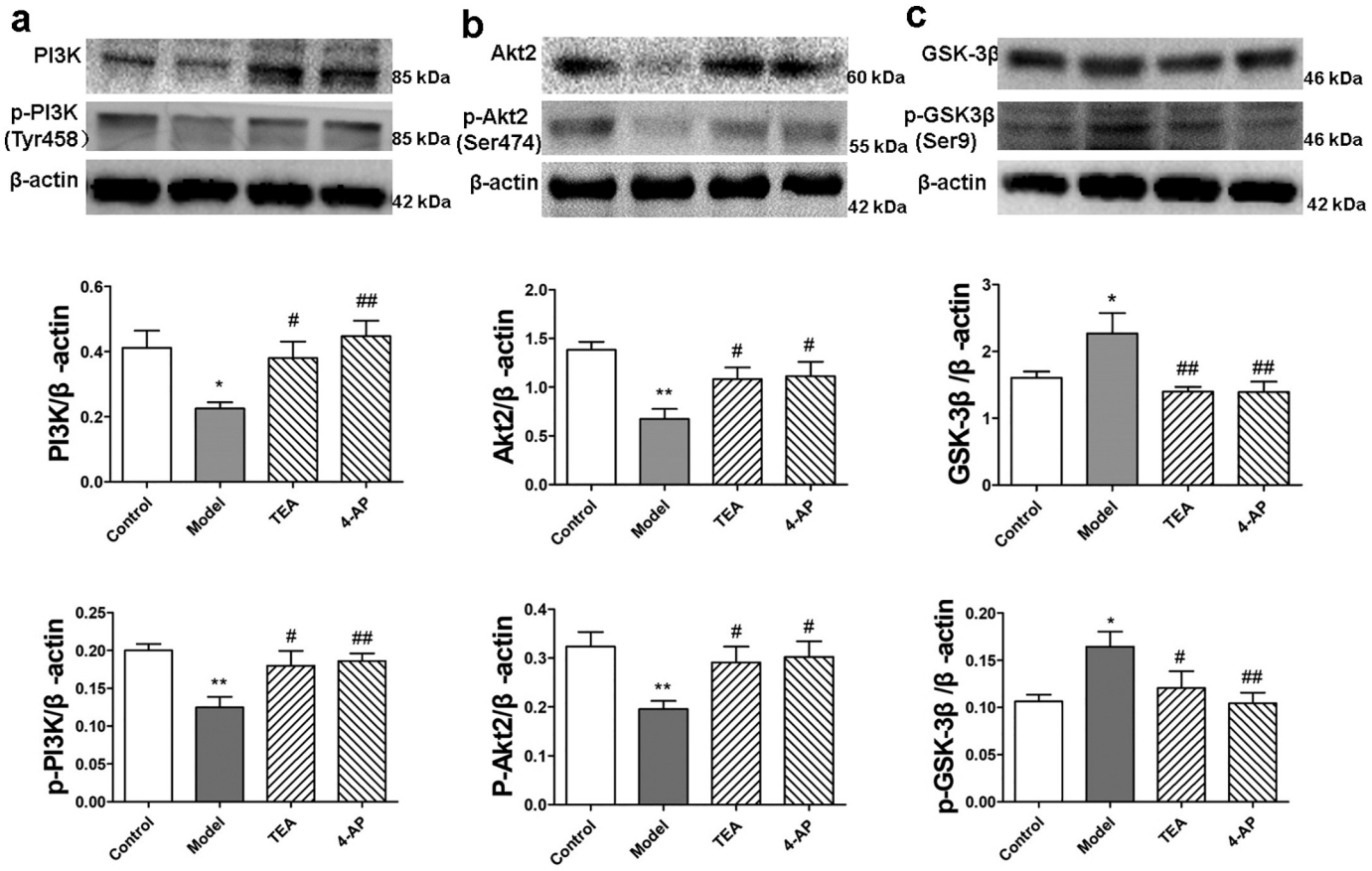

Fig. 7. TEA and 4-AP activate the PI3K/Akt signaling pathway in the hippocampus of STZ-induced diabetic mice. (a) Represent protein bands and quantification of PI3K and p-PI3K. (b) Represent protein bands and quantification of Akt2 and p-Akt2. (c) Represent protein bands and quantification of GSK-3β and p-GSK-3β. For p-GSK-3β, n = 4; for the remaining proteins, n = 5. The data are expressed as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, versus the control group; $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$, versus the model group.

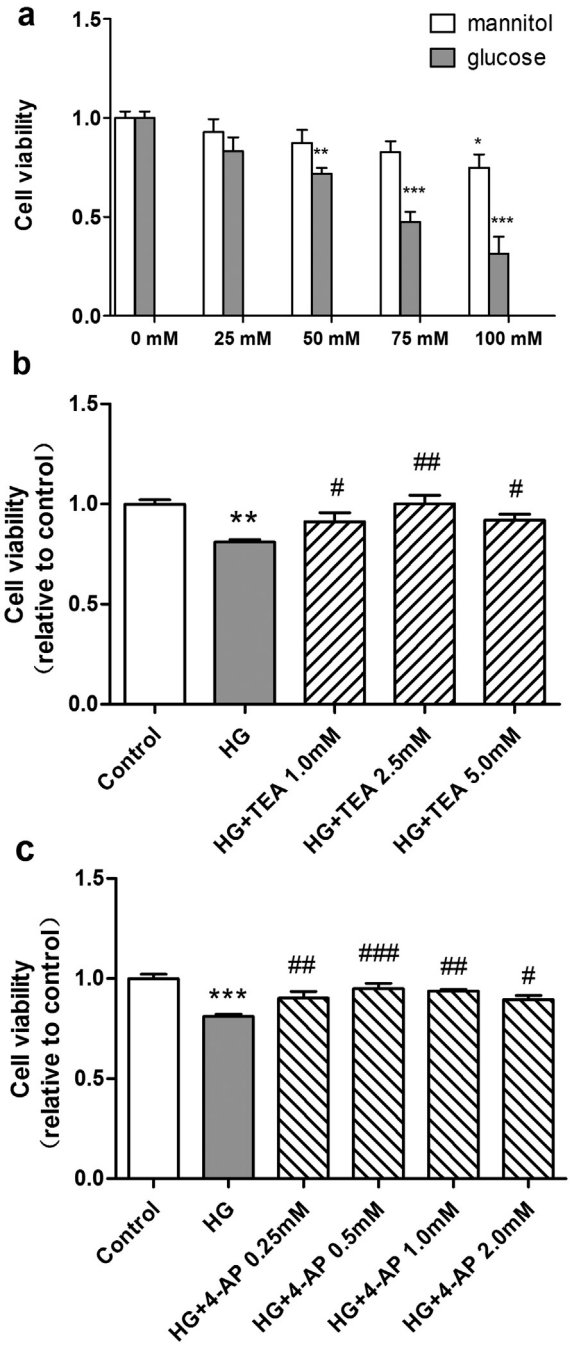

Fig. 8. The cell viability of primary cortical neurons. (a) Effects of different concentrations of glucose and mannitol on primary cortical neurons. The cell viability was measured by MTT assay. Cell viability was calculated by the ratio of OD value of each group to that of the group untreated glucose and mannitol. Data were expressed as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, versus 0 mM. $n = 3$; The effects of different concentrations of TEA (b) and 4-AP (c) on HG-injured primary cortical neurons. The cell viability was measured by MTT assay. Cell viability was calculated by the ratio of OD value of each group to that of the control group. Data were expressed as mean ± SEM. $^{**}P < .01$, $^{***}P < .001$, versus control; $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$, versus HG. $n = 4$.

0.5 mM 4-AP significantly increased SOD activity (Fig. 9a, $P < .01$) and decreased MDA levels (Fig. 9b, $P < .01$) compared with HG, while TEA and 4-AP alone did not affect the cultured cortical neurons.

3.10. TEA and 4-AP inhibit the upregulation of Kv channels and regulate the apoptosis-related proteins via the PI3K/Akt signaling pathway in the HG-induced primary cortical neurons

An increase in K^+ efflux mediated by delayed rectifier potassium channels functions as an apoptotic signal to trigger programmed cell death. However, little is known about whether other types of potassium channels are involved in the process of cell apoptosis. Therefore, we investigated the roles of delayed rectifier potassium channels and A-type channels in the cell apoptosis.

The protein levels of Kv2.1 and Kv4.2 were detected using Western blotting. In the present study, primary cortical neurons showed abnormal expression of Kv2.1 and Kv4.2 under HG conditions (Fig. 10a). However, treatments with TEA and 4-AP reversed the upregulation of Kv channels. The apoptosis-related proteins, such as cleaved caspase 3, Bcl-2 and Bax, were also detected in HG-stimulated primary cortical neurons. HG induced increased expression of cleaved caspase 3 and Bax, and decreased Bcl-2 expression (Fig. 10b). Compared with the HG group, TEA and 4-AP significantly increased the expression of anti-apoptotic protein Bcl-2 and decreased the expression of pro-apoptotic protein Bax; TEA markedly decreased the expression of cleaved caspase 3 while 4-AP had no effect on the expression of cleaved caspase 3.

LY294002 (hereafter called LY), a PI3K inhibitor, was used to further evaluate whether TEA and 4-AP exerted their anti-apoptosis effects on HG-induced primary cortical neuronal damage via the PI3K/Akt signaling pathway. As shown in Fig. 10c, the HG group exhibited a significantly decrease in the protein levels of PI3K, decreased p-Akt2 expression and increased protein levels of GSK-3β in cultured cortical neurons compared with the control group. Treatments with TEA and 4-AP reversed the HG-induced changes of intermediates in the PI3K/Akt/GSK-3β signaling pathway. In addition, LY partially abolished the effects of TEA and 4-AP. LY evidently reversed the activation of the PI3K/Akt signaling pathway and the decreased expression of Bax mediated by TEA and 4-AP.

4. Discussion

DM is associated with the increased risk of dementia (Kuo et al., 2018). However, the multifactorial pathogenesis of diabetes-associated cognitive dysfunction is not yet completely understood. Blocking specific Kv channels has been proposed as a promising strategy for the treatment of neurodegenerative diseases, while the underlying mechanisms remain to be clarified. In our present study, we tested whether the Kv channel blockers TEA and 4-AP exerted positive effects on several features of STZ-induced diabetic mice with cognitive decline and HG-induced rat primary cortical neurons: hippocampal and synaptic injuries, oxidative stress and apoptosis. We aimed to investigate the role of Kv channel in diabetes-associated cognitive dysfunction.

Kv channels have been implicated in neurological disorders such as AD and PD (Boscia et al., 2017; Rangaraju et al., 2015; Subramaniam et al., 2014; Yin et al., 2017). In the present study, the expressions of Kv2.1 and Kv4.2 were upregulated in vivo and in vitro, while TEA and 4-AP inhibited their upregulation. Kv channels blockers exert neuroprotective effects on neurodegeneration in vitro and in vivo. Blocking Kv channels thus suppresses K^+ efflux might prevent cell death and apoptosis (Leung, 2010; Shah and Aizenman, 2014). TEA reduces neuronal apoptosis induced by serum deprivation and Aβ (Yu et al., 1997; Yu et al., 1998). Similarly, our present study showed that TEA and 4-AP increased the cell viability of HG-stimulated primary cortical neurons, and participated in apoptosis by regulating apoptosis-related proteins both in STZ-induced diabetic mice and HG-induced primary cortical neurons, which will be mentioned in the subsequent paragraph. According to Gomes et al., the intraventricular injection of a selective A-type K^+ current blocker Tx3–1 facilitates the consolidation of short-term and long-term memory in object recognition tests and improves memory impairment in $A\beta_{25–35}$-treated mice (Gomes et al., 2013). In

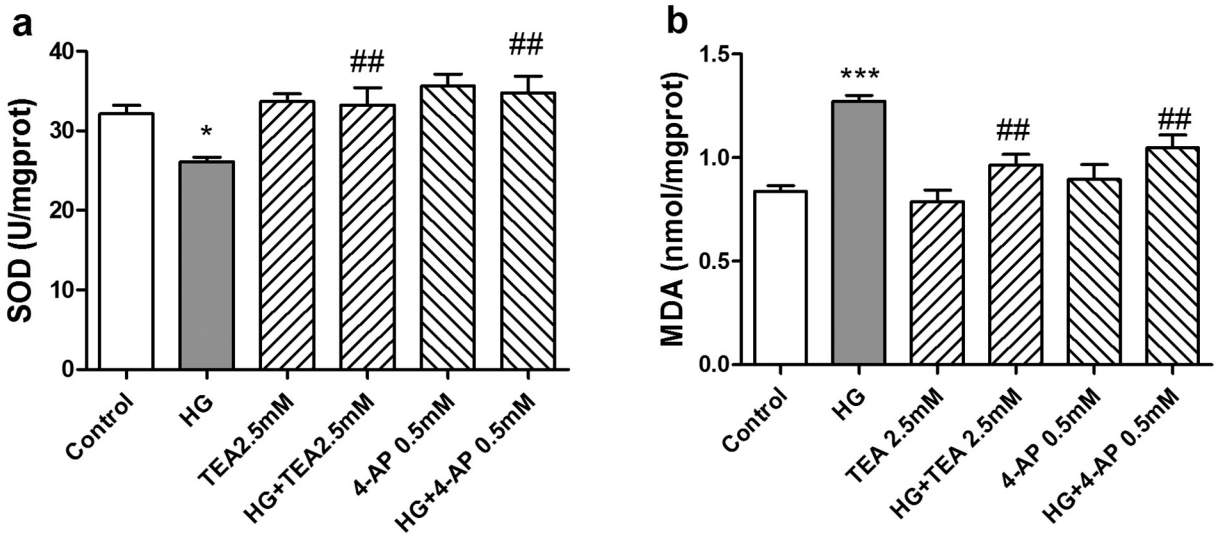

Fig. 9. Effects of TEA and 4-AP on SOD activity and MDA levels of HG-injured primary cortical neurons. After cultured for 7 days, primary cortical neurons were treated with potassium channel blockers TEA and 4-AP for another 48 h in the absence or presence of HG. Data were expressed as mean ± SEM. $^{*}P < .05$, $^{***}P < .001$, versus control; $^{\#\#}P < .01$, versus HG. n = 4.

addition, TEA and 4-AP have been proved to exert protection on 6-OHDA-induced PD (Haghdoost-Yazdi et al., 2011; Haghdoost-Yazdi et al., 2016; Haghdoost-Yazdi et al., 2017). In our study, 4-AP significantly decreased the FBG levels in STZ-induced diabetic mice, while TEA had little effect. Additionally, TEA and 4-AP ameliorated the spatial learning and memory deficits of diabetic mice. Interestingly, 4-AP exerted better effects on cognitive performance than TEA. Because 4-AP decreased the elevated FBG levels in STZ-induced diabetic mice, we observed the ultrastructure of pancreatic β cells by using TEM. In parallel with the FBG result, the ultrastructural examination of pancreatic β cells indicated that 4-AP, rather than TEA, increased the number of insulin secretory granules in pancreatic β cells.

The hippocampus is a key brain region responsible for learning and memory and is vulnerable to many diseases. Pathological abnormalities in the hippocampus, including neuronal and synaptic alterations, are well correlated with cognitive decline and are highly likely to induce the development of dementia (Sadeghi et al., 2016). Meanwhile, the hippocampus is a region which is particularly sensitive to changes in glucose homeostasis. Numerous studies have shown that DM has negative impacts on the hippocampus. Both in type 1 and type 2 diabetic rats, researchers have observed decreased number of pyramidal cells in the hippocampus (Abdel-Moneim et al., 2017; Zhang et al., 2017b). In the mice with cognitive impairments induced by an intracerebroventricular injection of streptozotocin, the synaptic density in the CA1 region of hippocampus is markedly decreased (Wang et al., 2018). Similarly, our studies reported neuronal and synaptic loss in the STZ-induced diabetic mice, while TEA and 4-AP increased the numbers of neurons and synapses and the thickness of the postsynaptic density in the hippocampus of STZ-induced diabetic mice. Based on these results, TEA and 4-AP relieved the hippocampal and synaptic injuries in the STZ-induced diabetic mice.

Oxidative stress plays an important role in the pathogenesis of neurodegeneration such as AD and diabetes-associated cognitive dysfunction (Hamed, 2017; Wojsiat et al., 2018). Oxidative stress is involved in the decreased cerebral glucose metabolism in AD and thus results in a disturbance in glucose homeostasis and neuronal damage (Markowicz-Piasecka et al., 2017). Furthermore, oxidative stress increases Aβ accumulation and tau phosphorylation, affecting the development and progression of AD (Ganguly et al., 2017). As shown in our previous study, HG-induced PC12 cells apoptosis is related to the increased oxidative stress. SOD decreases cellular injury by eliminating the oxygen free radicals in the body, playing an essential role in maintaining redox equilibrium. MDA, a marker of lipid peroxidation, indirectly indicates the intensity of cell damage induced by free radicals. The determination of MDA level is usually combined with the determination of SOD activities to evaluate the degree of oxidative stress. Based on the results of our in vivo studies, SOD activity was decreased and the MDA level was increased in the hippocampus of STZ-induced diabetic mice. However, the treatments with TEA and 4-AP increased SOD activity and decreased MDA levels. The in vitro studies also confirmed that TEA and 4-AP reversed the oxidative stress induced by HG in the primary cultured cortical neurons. These results suggested that TEA and 4-AP exerted antioxidant effect on diabetes-associated cognitive dysfunction.

The PI3K/Akt signaling pathway is known to be involved in the regulation of apoptosis and survival. Impaired insulin signaling affects the activity of the p85 regulatory subunit of PI3K, dephosphorylates Akt and activates GSK-3β. The increased activity of GSK-3β promotes the hyperphosphorylation of the microtubule associated protein tau, thereby forming intracellular neurofibrillary tangles (NFTs). The NFTs may induce oxidative stress and synaptic dysfunction that cause cognitive decline (Bitra et al., 2015). On other hand, the PI3K/Akt signaling also affects anti-apoptotic and pro-apoptotic members of Bcl-2 family, which control the release of cytochrome *c* (Jha et al., 2015; Ouyang et al., 2017). In the cytosol, cytochrome *c* complexes with caspase-9 and the adaptor Apaf-1 to form the apoptosome, activating the downstream executioner caspase-3 and subsequently leading to apoptosis (Kolb et al., 2017). In the present study, the blockade of Kv channels by TEA and 4-AP decreased apoptosis by regulating apoptosis-related proteins cleaved caspase 3, Bcl-2 and Bax, and activated the PI3K/Akt pathway in the STZ-induced diabetic mice and HG-stimulated primary cortical neurons. Treatments with TEA and 4-AP significantly decreased protein expression of the Kv channel subunits Kv2.1 and Kv4.2, increased the expression of PI3K, p-PI3K, Akt2 and p-Akt2, and downregulated the protein expression of GSK-3β and p-GSK-3β in the diabetic mice. The results from HG-stimulated primary cortical neurons were consistent with the data from the animal experiments. LY294002, a specific inhibitor of PI3K, was used to further identify the role of the PI3K/Akt signaling in diabetes-associated cognitive dysfunction,. LY partially abolished the effects of TEA and 4-AP. LY noticeably reversed the activation of the PI3K/Akt signaling and the decreased expression of Bax mediated by TEA and 4-AP. These findings suggested that TEA and 4-AP might exert neuroprotective effects on the HG-induced primary cortical neurons by activating the PI3K/Akt signaling pathway.

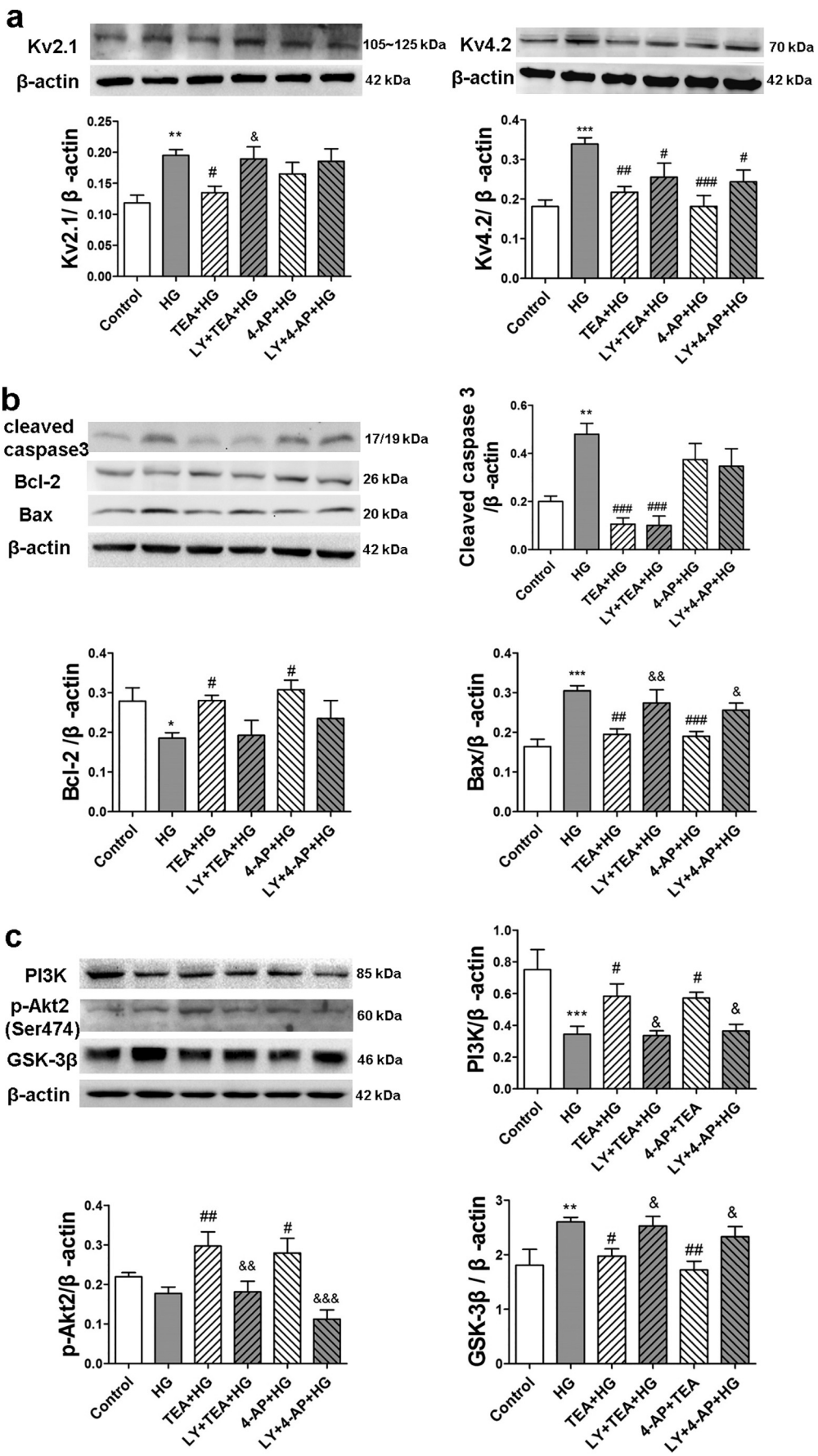

Fig. 10. Kv channel blockade prevents apoptosis and regulates the PI3K/Akt signaling pathway in the HG-induced primary cortical neurons. (a) Represent proteins bands and quantification of Kv channel subunits Kv2.1 and Kv4.2. For Kv2.1, $n = 5$; for Kv4.2, $n = 4$. (b) Represent proteins bands and quantification of apoptosis-related proteins cleaved caspase 3, Bcl-2 and Bax. For cleaved caspase 3 and Bcl-2, n = 4; for Bax, $n = 5$. (c) Represent proteins bands and quantification of PI3K, p-Akt2 and GSK-3β. For PI3K and GSK-3β, n = 5; for p-Akt2, $n = 6$. The data are expressed as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, versus Control; $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$, versus Model. $^{\&}P < .05$, $^{\&\&}P < .01$, $^{\&\&\&}P < .001$, versus TEA or 4-AP group without LY. LY represents the inhibitor of PI3K, LY294002.

There are still a few limitations in our study. First, considering the simpler operation, primary cultured cells were prepared from the embryos of SD rats rather than that of C57BL/6 J mice, although it is widely accepted to use different species in many literatures. Second, we used primary cortical neurons instead of hippocampal neurons in the cell experiment. Even our in vitro results were consistent with in vivo results, we have to admit that difference can exist between cortical neurons and hippocampal neurons. This may be related to the different expression of some molecules and receptors in these two brain regions, for example, the expression of Kv4.2 in the cortex is lower than hippocampus (Tsaur et al., 2001), and the density of NMDA receptors in the hippocampus is higher than cortex (Monaghan et al., 1988). In some cases, these differences may lead to different results in the cortex and hippocampus.

5. Conclusions

According to the results from our in vivo experiments, TEA and 4-AP ameliorated the cognitive deficits, improved the ultrastructures of pancreatic β cells, hippocampal neurons and synapses, decreased oxidative stress, blocked Kv2.1 and Kv4.2, prevented apoptosis and activated the PI3K/Akt/GSK-3β signaling pathway in the hippocampus. In vitro, Kv channels blockade by TEA and 4-AP increased the cell viability of HG-stimulated primary cortical neurons, reversed oxidative stress, and regulated apoptosis-related proteins through the PI3k/Akt signaling pathway. We conclude that the blockade of Kv channels by TEA and 4-AP ameliorated the diabetes-associated cognitive dysfunction via the PI3K/Akt pathway, suggesting an involvement of Kv channels in the pathogenesis of diabetes-associated cognitive impairments. Our results may raise the hope that the blockade of Kv channels represents a valuable therapeutic option for the treatment of diabetes-associated cognitive dysfunction.

The mechanisms by which Kv channels modulate diabetes-associated cognitive dysfunction are far from being completely elucidated. We will continue to investigate the alterations in delayed rectifying K^+ currents and A-type fast-inactivating K^+ currents, and elucidate their interactions with synaptic plasticity in HG-stimulated rat primary cortical neurons.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (81471031).

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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