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ARTICLE

Inhibition of endothelial nitric oxide synthase reverses the effect of exercise on improving cognitive function in hypertensive rats

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Abstract

Hypertension-induced endothelial dysfunction is associated with β-amyloid (Aβ) deposition, a typical pathology of Alzheimer's disease (AD). Endothelial nitric oxide synthase (eNOS) phosphorylation, impaired by phosphatidylinositol 3-kinase (PI3K)/protein kinase-B(Akt) pathway abnormalities in hypertensive rats, has a critical role in endothelial function. However, it is unknown whether eNOS participates in the hypertension-induced pathology of AD. In this study, we investigated the role of eNOS in Aβ deposition and cognitive function in stroke-prone spontaneously hypertensive (SHRSP) rats. Physical exercise was used as a promoter, and N^{ω}-nitro L-arginine methyl ester (L-NAME) was used as an inhibitor of eNOS to determine the effects of eNOS on SHRSP rats. Compared with Wistar Kyoto (WKY) rats, the hypertensive challenge caused cognitive impairment, decreased eNOS levels and increased amyloid precursor protein (APP), β-secretase, and Aβ levels in the cortex and hippocampus. Sixteen weeks of exercise lowered blood pressure (BP), promoted eNOS expression, ameliorated Alzheimer's pathology, and improved cognitive function in 29-week-old SHRSP rats. Furthermore, daily treatment with L-NAME reversed the beneficial effects of exercise on SHRSP rats. Exercise also decreased the protein levels of insulin-like growth factor-1 (IGF-1), PI3K, and phospho-Akt (p-Akt, ser473). In addition, long-term exercise increased the expression levels of IGF-1, PI3K, and p-Akt (ser473) in the brains of SHRSP rats. In conclusion, eNOS downregulation contributed to hypertension-induced Alzheimer pathology and cognitive impairment. Long-term exercise initiated in rats at a young age promoted eNOS expression and attenuated vascular-related Alzheimer's pathology via the IGF-1/PI3K/p-Akt pathway in SHRSP rats.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and is the most common cause of dementia in the elderly [1]. Aβ, a well-known neuronal toxin in AD, is derived from the sequential proteolytic cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase [2]. A previous study suggested that APP had functional roles in coagulation, adhesion, and inflammation in endothelial cells [3]. β-Secretase (also known as β-site APP-cleaving enzyme 1, BACE1) has been reported to be independently associated with cognitive deficits in AD [4]. Cognitive impairment can be prevented and treated by maintaining APP, BACE1, and Aβ levels in the brain.

Epidemiological data hint at a possible link between midlife hypertension and a predisposition to AD in later life [5]. Both postmortem and animal studies demonstrated that hypertension is associated with increased Aβ pathology and neurofibrillary tangles, which are neuropathologic hallmarks of AD [1, 5–10]. Hypertension can induce structural and functional damage to endothelial cells and the vascular system [11]; subsequently, it may lead to Aβ deposition in the brain parenchyma [1, 8]. Endothelial nitric oxide

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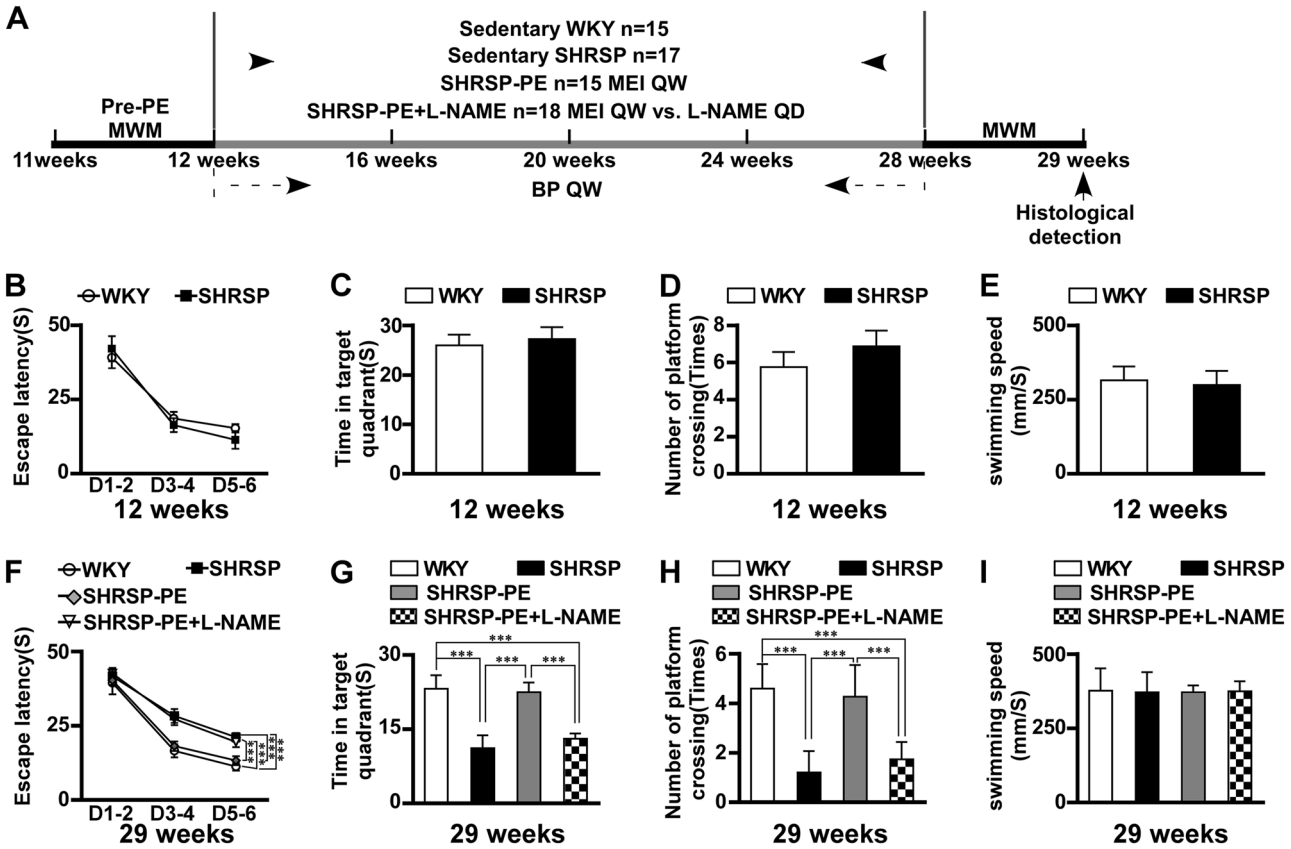


Fig. 1 Flow chart of the experimental protocol and Morris water maze (MWM) performance for each group. **a** Eleven-week-old WKY ($n = 15$) and SHRSP ($n = 50$) rats underwent pre-physical exercise (Pre-PE) and were tested using the MWM. SHRSP rats were randomly divided into three groups. Rats in the SHRSP-PE and SHRSP-PE+L-NAME groups were tested for their maximal exercise intensity (MEI) once a week (QW) for 16 weeks. Rats in the SHRSP-PE+L-NAME group were treated with L-NAME every day (QD) for 16 weeks. All the rats were subjected to BP QW measurement and MWM tests at 28 weeks. MWM performance for all of the rats was evaluated using the escape latency **b**, **f**, time in the target quadrant **c**, **g**, number of platform crossings **d**, **h**, and swimming speed **e**, **i**. The values are expressed as the mean ± SD, $^{***}p < 0.001$. WKY, Wistar Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; PE, physical exercise; L-NAME, N^{ω}-nitro L-arginine methyl ester

synthase (eNOS) is expressed exclusively in endothelial cells under basal conditions and it generates endothelial nitric oxide, a crucial signaling molecule responsible for maintaining vascular homeostasis [12]. In patients with hypertension, impaired eNOS expression and phosphorylation is common, even at a young age [13–15]. Furthermore, eNOS inhibition significantly increases APP expression and conversion to Aβ in the brain parenchyma by directly modulating Aβ, APP, and BACE1 levels in eNOS-deficient mice [3, 12]. However, the roles of eNOS in impaired cognitive function caused by Aβ deposition have not been well investigated in hypertension.

Physical exercise, a non-pharmacological approach, could protect against the development of AD in humans [16] and it also has beneficial effects on hypertension, such as delaying the onset of hypertension or lowering blood pressure (BP), even in subjects with resistant hypertension [17–19]. It was also shown that exercise can improve endothelial dysfunction, decrease inflammation, and reduce left ventricular hypertrophy, thus decreasing the risk of morbidity and mortality [17, 19–21]. In our previous study, we found that physical exercise had beneficial effects on functional recovery in ischemic stroke, and that insulin-like growth factor-1 (IGF-1) was involved in the effects of exercise through the Akt signaling pathway [22, 23]. IGF-1 has a beneficial role in maintaining endothelial function; on the other hand, lower IGF-1 levels are associated with a higher risk of endothelial dysfunction in hypertension [24]. IGF-1 can regulate vascular tone and vasorelaxation through eNOS activity by activating the phosphatidylinositol 3-kinase (PI3K pathway) in the aorta in hypertensive animals [15]. Furthermore, abnormal PI3K/Akt pathway activity in the cerebral cortex is related to impaired eNOS phosphorylation and activation in stroke-prone spontaneously hypertensive (SHRSP) rats [13], an animal model with hypertension, cerebral small vessel disease (CSVD), and Aβ plaques in the brain [7, 10]. Whether IGF-1/PI3K/

Akt participate in the effects of exercise on eNOS in the brains of hypertensive rats remains unknown.

To address the questions above, we aimed to investigate whether phospho-eNOS (p-eNOS) is involved in hypertension-related Alzheimer's pathology and cognitive impairment. To further clarify the effects of eNOS and the underlying mechanism, we used physical exercise as a promotor and N^{ω}-nitro L-arginine methyl ester (L-NAME) as an inhibitor of eNOS, and we analyzed the expression levels of APP, BACE1, Aβ, and IGF-1/PI3K/Akt in the brain regions primarily responsible for cognition in SHRSP rats.

Experimental procedures

Animal model

The Institutional Animal Ethical Committee of Sun Yat-Sen University authorized the experimental protocol and all procedures involving animals were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (Publication No. 80–23, revised 1996). The authors further attest that all efforts were made to minimize the number of animals used and their suffering. Experiments (Fig. 1a) were carried out on 11-week-old male SHRSP rats ($n = 50$) or age-matched normotensive Wistar Kyoto (WKY) rats ($n = 15$) (Vital River Laboratory Animal Technology Co. Ltd, Beijing, China). SHRSP rats were randomly assigned to the SHRSP group (sedentary control group, $n = 17$, 2 rats died during the experiment), the SHRSP-PE group (long-term exercise group, $n = 15$), or the SHRSP-PE+L-NAME group ($n = 18$, 3 rats died during the experiment). L-NAME (Sigma, N5751, CA, USA), an eNOS inhibitor, was administered orally (50 mg/L) every day from weeks 12–28 to the SHRSP-PE+L-NAME group [25, 26]. The rats were housed in an animal room at 24 °C ± 5 °C, 45–55% humidity, and a 12 h light/dark (08:00–20:00 h) cycle with sufficient food and water.

Exercise protocols

A 16-week exercise protocol with a wheel (21 cm in diameter, made in China) was implemented according to a previous study with modifications [21]. The maximal exercise intensity (MEI) was evaluated for each rat. Briefly, after 1 week of acclimation to wheel running, rats (12 weeks old) in the SHRSP-PE and SHRSP-PE+L-NAME groups were allowed to run on the wheel [22, 23] starting at a speed of 1 rev/min for 30 s. The speed was increased by 2 rev/min every 30 s until 85–90% of the expected MEI was achieved. After that, the speed increment was adjusted by 1 rev/min every 30 s. The highest intensity was recorded as the MEI, at which the rats were unable or unwilling to continue running. The whole evaluation process took 4–6 min, depending on the running potential of the individual animals. After determining the MEI, the rats were randomly assigned to the SHRSP-PE group or the SHRSP-PE+L-NAME group and subjected to exercise at 50% of the MEI (1 h/day, 5 days/week for 16 weeks). The running intensity was tested each week throughout the experiment. Body weights were measured at 29 weeks of age for the rats in all four groups. Resting systolic BP (SBP) and heart rate were measured by an indirect tail-cuff sphygmomanometer (ML866 Powerlab 4/30, AD Instruments Pty Ltd, Sydney, Australia) once a week in awake rats. The average of three measurements was used as the SBP reading.

Morris water maze test

The Morris water maze (MWM) test is a sensitive method to assess spatial learning and memory impairment. Before exercise and after 16 weeks of exercise, all rats in the four groups were tested in the MWM as previously described by Morris [27]. The experimental apparatus comprised a circular water tank containing water (23 ± 1 °C) at a depth of 20 cm, and the water was colored black. A platform (5 cm in diameter, 15 cm in height) was submerged below the water surface and placed at the midpoint of one quadrant. First, each rat received one training trial per day for 6 consecutive days. For each trial, the time for each rat to find the hidden platform was recorded. Rats that found the platform remained on the platform for 10 s and were then returned to their cages. If a rat failed to locate the platform within 60 s, escape was assisted, and the escape latency was recorded as 60 s. The latency to escape from the water maze (finding the submerged escape platform) was calculated for each trial. On day 7, the probe test was carried out by removing the platform and allowing each rat to swim freely for 60 s. The time spent swimming in the target quadrant where the platform was located during the hidden platform training and the number of times the rat crossed over the platform site were recorded. The mean data from the daily training trial tests and the data from the tests on the last day were used for statistical analyses.

Tissue preparation for histochemistry

Rats were anesthetized with sodium pentobarbital (200 mg/kg, intraperitoneal (i.p.)) after completing the MWM ($n = 5$ per group) and perfused transcardially with 0.9% saline at 4 °C, followed by 4% paraformaldehyde in phosphate buffer (0.1 mol/L, pH 7.4). The brains were removed, fixed in the aforementioned fixative solution for 8 h at 4 °C, and then immersed sequentially in 20% and 30% sucrose until

sinking occurred. Coronal sections (10 μm thick) were cut on a cryostat (CM1900; Leica, Heidelberger, Germany) from the bregma – 3.0 to – 5.0 mm and used for immunostaining.

Immunostaining

Eight consecutive sections at 250 μm intervals from the bregma – 3.0 to – 5.0 mm were used for immunostaining or double-labeled immunofluorescence staining. The sections were pretreated for 5 min with hot (85 °C) citrate buffer (0.01 mol/L, pH 6.0) for antigen retrieval, followed by 5% normal goat serum for 1 h at room temperature. Next, the sections were incubated with the following primary antibodies overnight at 4 °C: mouse anti-BACE1 (1:200, Millipore, MAB5308, MA, USA)/rabbit anti-eNOS (1:200, Novus Biological, NB300-500, CO, USA), rabbit anti-BACE1 (1:100, Abcam, ab10716, Cambridge, UK)/mouse anti-Neun (neuronal marker, 1:400, Millipore, MAB377), mouse anti-glial fibrillary acidic protein (GFAP, 1:100, Abcam, ab10062), rabbit anti-App (1:300, Abcam, ab15272), or rabbit anti-β $Amyloid_{1\text{-}42}$ (1:300, Abcam, ab201060). After rinsing with phosphate-buffered saline (PBS) three times for 5 min each, the sections were incubated with rabbit/mouse secondary antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. The sections were then mounted in fluorescent mounting medium with DAPI (4',6-diamidino-2-phenylindole) (Sigma) or labeled with 3,3-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark); next, images were taken using a microscope (BX51; Olympus). Negative control sections were incubated with PBS instead of primary antibodies and showed no positive signals.

Western blotting

Rats ($n = 5$) were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) and perfused intracardially with 50 ml of ice-cold 0.9% saline. The cortex and hippocampus of the brain were rapidly dissected and homogenized in a protein extraction reagent (Thermo, Pierce Biotechnology, USA) with complete protease inhibitor cocktail (Thermo, Pierce Biotechnology). Equal amounts of protein from each sample were separated on 8% (for subsequent incubation with p-eNOS/App antibodies), 10% (for subsequent incubation with BACE1/PI3K/ phospho-Akt/T-Akt antibodies), or 15% (for subsequent incubation with IGF-1 antibody) sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore). After blocking with 5% skimmed milk in tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature, the membranes were incubated with rabbit anti-p-eNOS (Ser1177) (1:1000, Cell Signaling Technology, 9570 s), rabbit anti-App (1:1000, Abcam, ab15272), mouse anti-BACE1 (1:200, Millipore, MAB5308), mouse anti-IGF-1 (1:500, Millipore, 05-172), rabbit anti-PI3K (1:1000, Cell Signaling Technology, 3358), rabbit anti-phospho-Akt (Ser473) (1:1000, Cell Signaling Technology, 4060), rabbit anti-Akt (1:1000, Cell Signaling Technology, 9272), or mouse anti-β-actin (1:1000, Sigma) overnight at 4 °C. The membranes were then washed in TBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (1:1000, Bioworld, USA). Immunoreactivity was visualized using the enhanced chemiluminescence method.

Quantification of Aβ by enzyme-linked immunosorbent assay

After intracardial perfusion with 50 mL of ice-cold 0.9% saline, the brains ($n = 5$) were used to measure cortex and hippocampus Aβ levels by enzyme-linked immunosorbent assay (ELISA). Cortices and hippocampi were immediately isolated on ice, frozen, and stored at – 80 °C; then, they were homogenized separately at 5 mL/g in ice-cold homogenization buffer (containing 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, and 0.6% sodium dodecyl sulfate) with complete protease inhibitor cocktail tablets (Thermo, Pierce Biotechnology). The resulting lysates were centrifuged at $250{,}000 \times g$ and 4 °C for 30 min. The supernatants were collected and stored at – 80 °C.

Total $A\beta_{x\text{-}42}$ levels were quantified by ELISA assay using the colorimetric Beta Mark $A\beta_{x\text{-}42}$ ELISA kit (Covance, 842401, New Jersey, USA) according to the manufacturer's instructions. The $A\beta_{x–42}$ ELISA kit is specific for the $x – 42$ isoforms of Aβ. $A\beta_{x–42}$ levels were calculated by comparison with a standard curve generated using the $A\beta_{x–42}$ standard. The standard curve was linear in the 0–250 pg/mL range and $R^2 = 0.99$. Each Aβ standard was run in duplicate and each experimental sample was run in triplicate. Serial dilutions of the samples were used for measurements in the linear range. The $A\beta_{x–42}$ levels of the cortices and hippocampi were expressed as pg/mg of the wet tissue weights.

Image analysis and quantification

All histological images captured at the same exposure were analyzed with Image-Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD, USA) and densitometric analyses for immunoblotting were performed using Quantity One software (Bio-Rad, Hercules, CA, USA) by one researcher who was blinded to the animal group assignments.

Table 1 General characteristics

	WKY	SHRSP	SHRSP-PE	SHRSP-PE+L-NAME
Number	15	15(survival)	15	15(survival)
Body weight (g, 29 week)	320 ± 10$^{\#}$	313 ± 8$^{\#}$	309 ± 11$^{\#}$	290 ± 12
BP (mmHg, 12 week)	126 ± 5	178 ± 7***	179 ± 5***	182 ± 4***
BP (mmHg, 29 week)	136 ± 4	203 ± 5***	146 ± 6$^{\#\#\#}$	201 ± 11***
Heart rate (bpm, 29 week)	346 ± 7	409 ± 4***	402 ± 8***	407 ± 5***
50% MEI (rev/min, 28 week)	-	-	15 ± 3$^{\#}$	10 ± 3

Values are means ± SD. ***$p < 0.001$, significant difference from WKY group, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, significant difference from SHRSP-PE+L-NAME group

Statistical analysis

All data are presented as the mean ± SD. The BP and escape latency results were compared using repeated-measures analysis of variance (ANOVA). Results for the time spent in the target quadrant, number of platform crossings, western blotting assays, and ELISA assays were analyzed using one-way ANOVA, followed by Bonferroni's test for the three groups. Statistical analyses were performed using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

General characteristics

Body weights were significantly lower in the SHRSP-PE +L-NAME group than in the WKY, SHRSP, and SHRSP-PE groups at 29 weeks (Table 1; $p < 0.05$). The resting heart rate was higher in the SHRSP group than in the WKY group at week 29 (Table 1; $p < 0.001$), but no significant differences were found among the SHRSP, SHRSP-PE, and SHRSP-PE+L-NAME groups. BP was much higher in all three SHRSP groups than in the WKY group at both week 12 and 29 (Table 1; $p < 0.001$); furthermore, exercise significantly lowered BP at week 29 (Table 1; $p < 0.001$). At the end of the experiment, the 50% MEI in the SHRSP-PE and SHRSP-PE+L-NAME groups was significantly different (Table 1; $p < 0.05$).

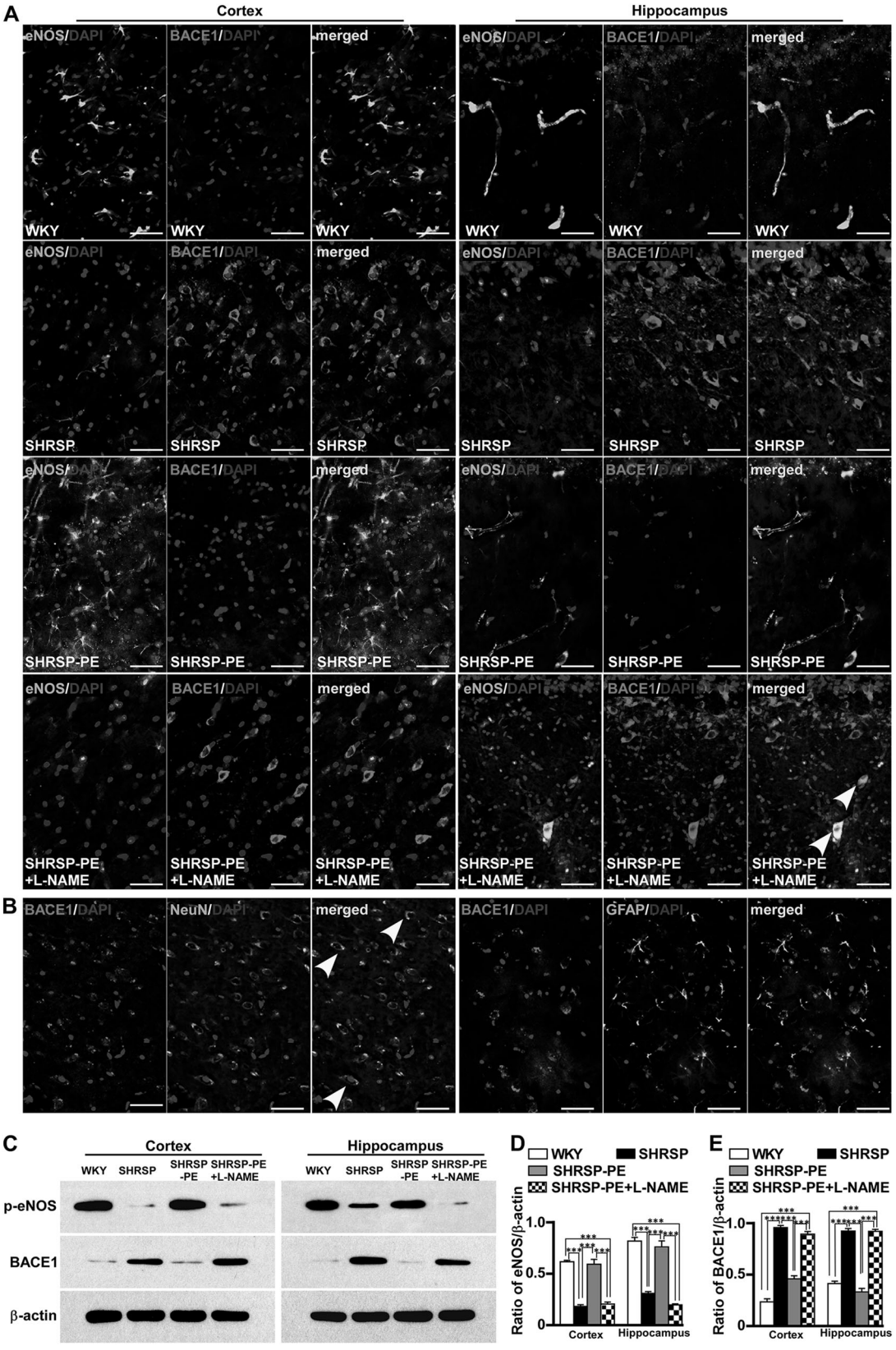
Long-term exercise improved cognitive function in SHRSP rats

As shown in Fig. 1b,c,d, there were no significant differences between the 12-week-old WKY rats and SHRSP rats in the MWM test. In 29-week-old rats (Fig. 1f), repeated-measures ANOVA revealed a significant interaction between group and time effects on the escape latency time in the training sessions of the MWM ($F_{(6, 112)} = 28.392$, $p < 0.001$). Further analysis using one-way ANOVA followed by Bonferroni's test for all four groups revealed that the escape latency time was significantly longer for the SHRSP group than the WKY group at days 3–4 and days 5–6 (Fig. 1f; $p_{adjust} < 0.001$, $p_{adjust} < 0.001$). Physical exercise shortened the escape latency time for days 3–4 and days 5–6 (Fig. 1f; $p_{adjust} < 0.001$, $p_{adjust} < 0.001$); in contrast, L-NAME prolonged the escape latency time for days 3–4 and days 5–6 (Fig. 1f; SHRSP-PE+L-NAME vs. WKY, $p_{adjust} < 0.001$, $p_{adjust} < 0.001$; SHRSP-PE+L-NAME vs. SHRSP-PE, $p_{adjust} < 0.001$, $p_{adjust} < 0.001$). In the probe test session, the time spent in the target quadrant and the number of platform crossings were lower in the SHRSP group than in the WKY group (Fig. 1g,h; $p < 0.001$, $p < 0.001$). Exercise significantly increased time spent in the target quadrant and the number of platform crossings (Fig. 1g,h; $p < 0.001$, $p < 0.001$), but L-NAME reversed the effects of physical exercise (Fig. 1g,h; SHRSP-PE+L-NAME vs. WKY, $p < 0.001$, $p < 0.001$; SHRSP-PE+L-NAME vs. SHRSP-PE, $p < 0.001$, $p < 0.001$). There were no differences in swimming speed for the groups (Fig. 1e,i).

Long-term exercise upregulated eNOS expression and downregulated BACE1 expression in the SHRSP cortex and hippocampus

Immunostaining (Fig. 2a,b) and immunoblotting (Fig. 2c) were performed to examine the expression of eNOS and BACE1 in the cortex and hippocampus. The protein levels of p-eNOS (Fig. 2c,d) in the cortex ($p < 0.001$) and hippocampus ($p < 0.001$) were significantly lower in SHRSP rats than in WKY rats. Compared with that in the SHRSP group (Fig. 2c,d), p-eNOS expression in the SHRSP-PE group was upregulated in the cortex ($p < 0.001$) and hippocampus ($p < 0.001$). L-NAME, an eNOS inhibitor (Fig. 2c,d), reduced p-eNOS expression in the cortex (vs. WKY, $p < 0.001$; vs. SHRSP-PE, $p < 0.001$) and hippocampus (vs. WKY, $p < 0.001$; vs. SHRSP-PE, $p < 0.001$).

Because of the critical role of BACE1 in APP amyloidogenesis [4], we measured the cortex and hippocampus


A
Cortex
Hippocampus
eNOS/DAPI
BACE1/DAPI
merged
WKY
SHRSP
SHRSP-PE
SHRSP-PE +L-NAME
B
BACE1/DAPI
NeuN/DAPI
GFAP/DAPI
C
Cortex
Hippocampus
WKY
SHRSP
SHRSP -PE
SHRSP-PE +L-NAME
p-eNOS
BACE1
β-actin
D
WKY
SHRSP
SHRSP-PE
SHRSP-PE+L-NAME
Ratio of eNOS/β-actin
1.0
0.5
0
Cortex
Hippocampus
E
Ratio of BACE1/β-actin

◀ **Fig. 2** eNOS and BACE1 expression determined by immunostaining and immunoblotting of the cortices and hippocampi from each group. **a** Double-immunofluorescence labeling of eNOS and BACE1 in the cortices and hippocampi from each group. **b** Cellular localization of BACE1 in the cortices and hippocampi from SHRSP rats. **c** eNOS and BACE1 immunoblotting. Protein lysates were isolated from the cortices and hippocampi from each group. **d**, **e** Quantification of the eNOS and BACE1 immunoblotting ($n = 5$). The values are expressed as the mean ± SD, $^{***}p < 0.001$. WKY, Wistar Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; PE, physical exercise; L-NAME, N^{ω}-nitro L-arginine methyl ester. Scale bar = 25 μm

levels of BACE1 for each group (Fig. 2c,e). BACE1 expression was significantly different in the cortex (SHRSP vs. WKY, $p < 0.001$; SHRSP-PE vs. SHRSP, $p < 0.001$; SHRSP-PE+LNAME vs. WKY, $p < 0.001$; SHRSP-PE +LNAME vs. SHRSP-PE, $p < 0.001$) and hippocampus (SHRSP vs. WKY, $p < 0.001$; SHRSP-PE vs. SHRSP, $p < 0.001$; SHRSP-PE+LNAME vs. WKY, $p < 0.001$; SHRSP-PE+LNAME vs. SHRSP-PE, $p < 0.001$). To further determine the expression pattern of BACE1, double-labeled immunofluorescence staining was performed using antibodies against BACE1, eNOS, and cell-type-specific markers (NeuN and GFAP). Double staining showed that there were more BACE1-positive cells and fewer eNOS-positive cells in the SHRSP and SHRSP-PE+L-NAME groups; in contrast, there were fewer BACE1-positive cells and more eNOS-positive cells in the WKY and SHRSP-PE groups. Several BACE1/eNOS-positive cells were detected (Fig. 2a). Furthermore, most of the BACE1-positive cells were co-labeled with NeuN (Fig. 2b), but few of the BACE1-positive cells were co-labeled with GFAP (Fig. 2b).

Long-term exercise ameliorated APP and Aβ deposition in the cortex and hippocampus of SHRSP rats

Marked positivity for anti-APP antibodies was observed in the cortex and hippocampus (Fig. 3a). APP expression levels (Fig. 3b,d) in the SHRSP cortex ($p < 0.001$) and hippocampus ($p < 0.001$) were markedly increased. Exercise decreased the APP expression levels in the cortex ($p < 0.001$) and hippocampus ($p < 0.001$).

To confirm Aβ deposition, brain tissue was processed for immunostaining (Fig. 3c) and ELISA assays (Fig. 3e). Aβ deposition (Fig. 3e) in the cortex ($p < 0.001$) and hippocampus ($p < 0.001$) was significantly higher in the SHRSP group than in the WKY group. Exercise decreased Aβ deposition in the cortex ($p < 0.001$) and hippocampus ($p < 0.001$).

L-NAME, however, weakened the effects of exercise on APP expression (Fig. 3b,d) and Aβ deposition (Fig. 3e) in the cortex (SHRSP-PE+LNAME vs. WKY, $p < 0.001$, $p < 0.001$, respectively; SHRSP-PE+LNAME vs. SHRSP-PE, $p < 0.001$, $p < 0.001$, respectively) and hippocampus (SHRSP-PE+LNAME vs. WKY, $p < 0.001$, $p < 0.001$, respectively; SHRSP-PE+LNAME vs. SHRSP-PE, $p < 0.001$, $p < 0.001$, respectively).

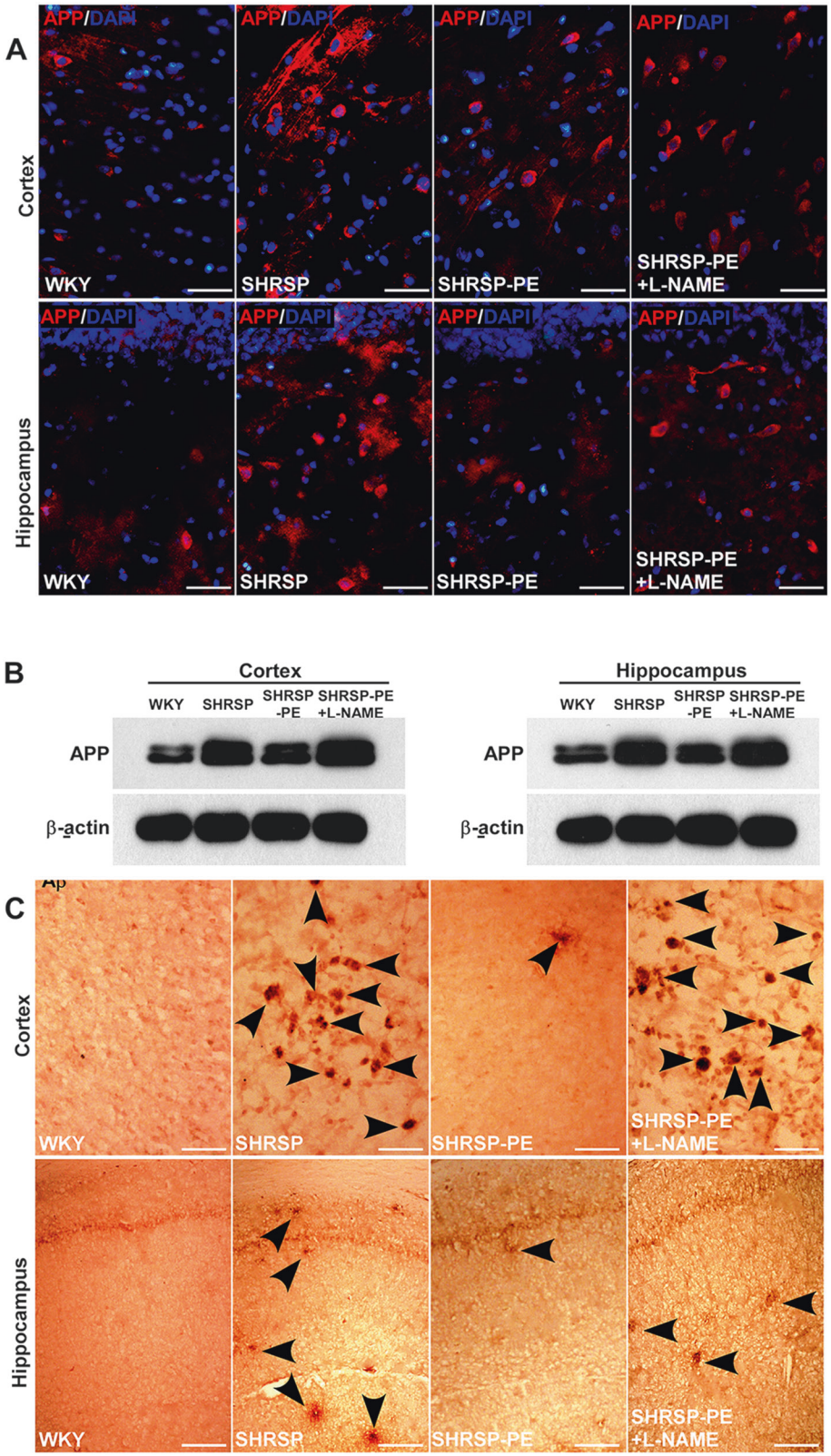
Long-term exercise promoted IGF-1 expression and activated the PI3K/Akt pathway in the cortex and hippocampus of SHRSP rats

IGF-1-positive cells were detected in the cortex and hippocampus (Fig. 4a), and IGF-1 protein levels (Fig. 4b,c) in the cortex ($p < 0.001$) and hippocampus ($p < 0.001$) were significantly lower in the SHRSP group than in the WKY group. Exercise increased the IGF-1-positive cell count in the cortex ($p < 0.001$) and hippocampus ($p < 0.001$). Activation of the serine/threonine kinase Akt leads to eNOS phosphorylation and activation. Therefore, we also investigated p-Akt levels in the cortex and hippocampus (Fig. 4b). The western blotting results indicated significantly lower p-Akt (Ser473) levels in the SHRSP rats than in the WKY rats and equal total Akt protein levels (Fig. 4b,e; $p < 0.001$). As Akt is located downstream of PI3K, we also assessed PI3K protein levels by western blotting; PI3K levels were significantly lower in SHRSP rats than in WKY rats (Fig. 4b,d; $p < 0.001$). Exercise increased the ratio of p-Akt (Ser473) to total Akt and the PI3K protein levels (Fig. 4d,e; $p < 0.001$, $p < 0.001$, respectively).

Discussion

Our results demonstrated that hypertension induced endothelial dysfunction, amyloid pathology and cognitive impairment. Long-term exercise positively influenced the effects of hypertension, and L-NAME reversed these beneficial effects of exercise; these results indicate that the up-regulation of eNOS expression by exercise might have a key role in improving cognitive function and attenuating amyloid pathology. Furthermore, the effects of exercise on eNOS expression occurred through the IGF-1/PI3K/Akt pathway.

Chronic hypertension is the top risk factor for both ischemic and hemorrhagic stroke, which lead to neurologic dysfunction or even death [28, 29]. Consistent with the results of a previous study [29], two rats died from ischemia and three rats died from hemorrhage in the SHRSP and L-NAME groups in our study. Hypertension induces chronic brain hypoperfusion and cerebral vascular remodeling, including arterial wall thickening accompanied by decreased lumen size [30], as well as endothelial dysfunction, which is associated with the down-regulation of eNOS expression [31, 32]. In a recent study, we analyzed the


A
Cortex
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SHRSP-PE
+L-NAME
B
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Hippocampus
WKY
SHRSP
SHRSP
-PE
SHRSP-PE
+L-NAME
APP
β-actin
C
Cortex
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SHRSP-PE
SHRSP-PE
+L-NAME

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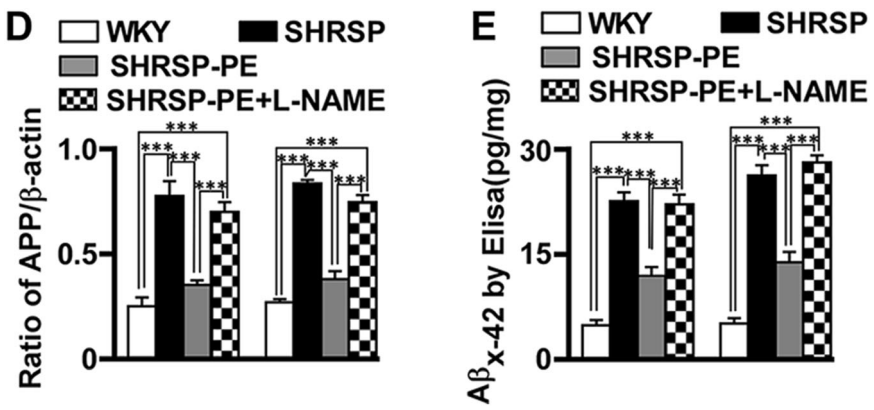

Fig. 3 APP and Aβ expression determined by immunostaining, immunoblotting, and ELISA in the cortices and hippocampi from each group. **a**, **b** Immunostaining and immunoblotting to determine APP expression levels in the cortex and hippocampus. **c** Immunostaining to show Aβ deposition in the cortex and hippocampus. **d** Quantification of the APP immunoblotting ($n = 5$). **e** Quantification of Aβ deposition by ELISA assay in the cortices and hippocampi from each group ($n = 5$). The values are shown as the mean ± SD, ***$p < 0.001$. L-NAME, N^{ω}-nitro L-arginine methyl ester; PE, physical exercise; SHRSP, stroke-prone spontaneously hypertensive rats; WKY, Wistar Kyoto rats. Scale bar = 25 μm

impact of chronic hypertension on eNOS expression and found marked endothelial dysfunction in the cerebral cortex and hippocampus areas, which are specifically involved in controlling important cognitive functions, such as learning and memory [7, 33, 34]. Under normal conditions, the brain endothelium, the main component of the blood–brain barrier, does not allow the free bi-directional exchange of polar solutes, such as Aβ, between the blood and the brain [35]. However, in chronic hypertension, endothelial dysfunction can cause the activation of several mechanisms of brain damage that favor Aβ production and/or Aβ accumulation in the brain [1, 3, 8, 12]. In a study by Bueche et al. [7], the authors found parenchymal Aβ deposition in the cortical regions (69%), basal ganglia (44%), hippocampus (31%), thalamus (6%), and corpus callosum (6%), and the age of the SHRSP rats had a significant effect on Aβ deposition. In our present study, we determined the parenchymal Aβ deposition in the main cognitive regions. We demonstrated that hypertension alters endothelial function by inhibiting p-eNOS expression and induces brain damage by increasing Aβ deposition in an animal model; these results corroborate the epidemiological and molecular data obtained in previous studies [1, 8, 36]. As previously reported, BACE1 was predominantly expressed in neurons, with little expression in astrocytes, in our current study. This cellular localization of BACE1 is similar to that seen in AD and ischemic brains [2, 37]. It is interesting that Aβ deposition in the brain was observed in the areas that are first involved in the onset of AD neuro-degeneration, such as the cortex and hippocampus, in the hypertensive rats in our study [1, 8]. Moreover, ELISA assays showed that Aβ deposition in these cerebral areas was higher in the hypertensive rat brains than in the normotensive rat brains and could cause cognitive impairment during hypertension. Although Aβ deposition in the hypertensive brain has been extensively studied in animal models and humans [1, 7, 8, 36], there has been no research on the involvement of APP and BACE1 in hypertension. APP can have functional roles in coagulation, adhesion, and inflammation [3, 12]. Moreover, BACE1 may contribute to memory deficits via Aβ-dependent and Aβ-independent pathogenic mechanisms in AD by regulating the cAMP/PKA/CREB pathway [4]. We observed the up-regulation of APP and BACE1 in brain tissue, indicating that APP and BACE1 may also contribute to cognitive impairment in hypertension. Furthermore, eNOS-derived NO promotes the prevention of Aβ accumulation by modulating BACE1 and APP expression in the neuronal tissue of mice [3, 12, 38]. Thus, inhibiting hypertension-induced eNOS expression may contribute to Aβ deposition via endothelial dysfunction, as well as increased APP and BACE1 expression.

Exercise is an important stimulus in the prevention of endothelial dysfunction [31]. According to previous studies [15, 19, 21, 31, 39], low (30% of the maximal aerobic velocity) and moderate (55% of the maximal aerobic velocity) but not high (80% of the maximal aerobic velocity) exercise intensity has beneficial effects on endothelium-dependent vasodilatation and eNOS expression in hypertensive rats. Niwa et al. [40] demonstrated that exercise induces neurogenesis in SHRSP rats. Most studies propose that the effects of exercise on hypertension are associated with lowing BP and improving endothelial function [15, 39]. Long-term moderate exercise was used in our study as a non-drug treatment for hypertension and proved to be an effective method for improving cognitive function. Our previous and present studies have shown that exercise can increase IGF-1 expression in the brains of ischemic and hypertensive rats [15, 22, 23]. In the present study, we observed a significant decrease in IGF-1 expression levels in the same injured cerebral areas.

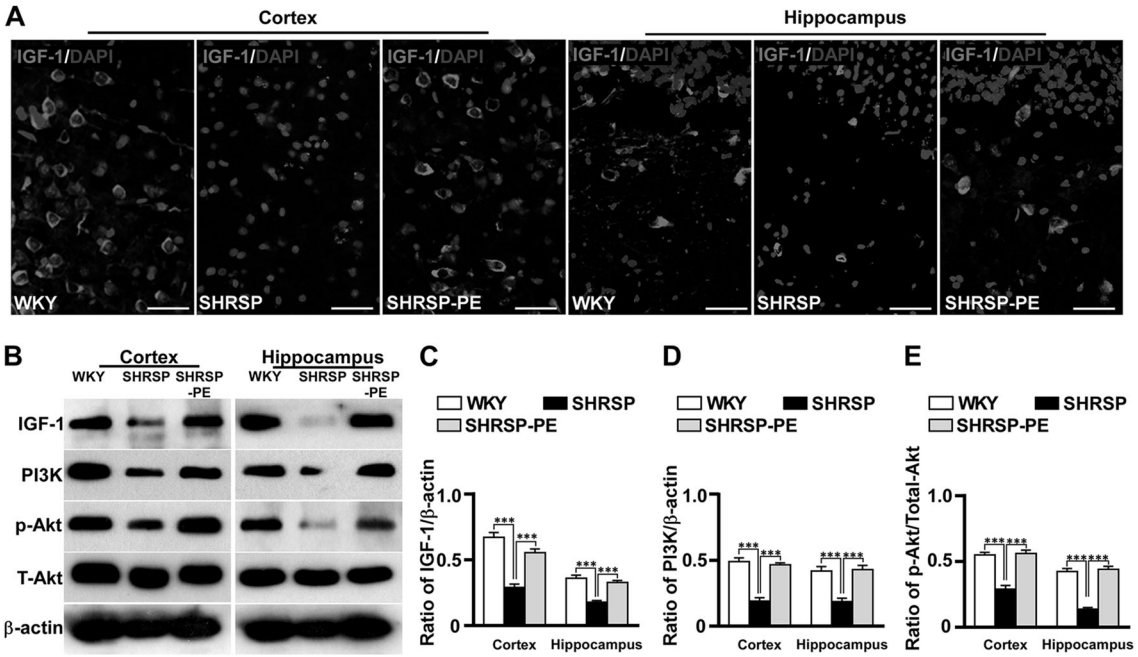

Fig. 4 IGF-1 expression determined by immunostaining and IGF-1/PI3K/p-Akt/T-Akt protein levels determined by immunoblotting in the cortex and hippocampus. **a** Immunostaining of IGF-1-positive cells in the cortex and hippocampus. **b** Immunoblotting to determine IGF-1/PI3K/p-Akt/T-Akt protein levels in the cortex and hippocampus. **c**–**e** Quantification of the IGF-1/PI3K/p-Akt/T-Akt immunoblotting ($n = 5$). The values are the mean ± SD. PE, physical exercise; SHRSP, stroke-prone spontaneously hypertensive rats; WKY, Wistar Kyoto rats. Scale bar = 25 μm

Lower IGF-1 levels are associated with a higher risk of endothelial dysfunction in the pathophysiology of hypertension [15]. In addition, IGF-1 has a regulatory role in the maintenance of endothelial function and can modulate normal vasorelaxation by binding to its receptors and further activating the PI3K/Akt pathway, which phosphorylates eNOS [14, 15]. In hypertension, exercise elicits eNOS-mediated vasorelaxation by increasing insulin/IGF-1 levels via PI3K activation [15]. We observed an increase in eNOS expression levels in brain tissue after exercise. Interestingly, Aβ deposition in the same areas was significantly decreased in hypertensive rats and cognitive improvement was observed after 16 weeks of wheel running. In contrast, the inhibition of eNOS by L-NAME significantly reversed the protective effects of exercise on both Alzheimer's pathology and cognitive function. We demonstrated that exercise-mediated eNOS upregulation can contribute to a decrease in Aβ deposition by protecting the endothelium [1, 8, 19, 21, 39].

The study has several limitations. First, direct evidence for the link between endothelial dysfunction and amyloid pathology is not fully investigated. Second, brain pathologies (e.g., bleeding, infarcts, lacunes, and white matter lesions) in the SHRSP rats were not studied. Lastly, WKY rats subjected to exercise or L-NAME treatment and SHRSP rats subjected to L-NAME treatment were not used to further prove our conclusions. Our future studies will focus on a detailed understanding of the links among hypertension, CSVD, and amyloid pathology; the duration and intensity of exercise on cognitive improvement in SHRSP rats; and different effects of the same exercise regimen on different hypertensive rat models.

Conclusions

In conclusion, our data revealed that a hypertensive vascular challenge leading to AD pathology and cognitive impairment was related to Aβ deposition and APP and BACE1 expression. Although we do not have data regarding the exact role of Aβ deposition and APP and BACE1 expression in hypertensive rat behavior, based on previous studies and the present study, we inferred that Aβ deposition and APP and BACE1 expression have a combined effect on hypertension. Our study demonstrated that exercise induced eNOS expression by regulating the IGF-1/PI3K/Akt pathway. This process could effectively improve cognitive function by modulating APP and BACE1 expression and reducing Aβ deposition. The benefits of long-term exercise in the prevention of hypertension could be substantial, not only to decrease BP and protect the vascular system but also to prevent or delay the sequelae of hypertension.

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Author contributions LZ, HZ, LL, JL, TJ, XP, and CX carried out the experiments. HZ performed the statistical analysis. LZ drafted the manuscript. XH and ZP participated in the design of the study. XH conceived the study, participated in the design and coordination, and helped to draft the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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