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Effects of α-lipoic acid on chronic cerebrovascular hypoperfusion in an animal model of vascular dementia

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Abstract. – OBJECTIVE: Given the aging population, the treatment of vascular dementia (VaD) is becoming increasingly important. The antioxidant α-lipoic acid (α-LA) protects against neurodegeneration in VaD, but the underlying mechanisms remain unclear. Hence, we aimed to identify the effects of α-LA on cognitive function following chronic cerebrovascular hypoperfusion in a VaD animal model.

MATERIALS AND METHODS: Mice were categorized into the sham, bilateral common carotid artery stenosis (BCAS), or BCAS + α-LA group. The BCAS + α-LA group was intraperitoneally injected (100 mg/kg) once daily with α-LA for 4 weeks after BCAS surgery, while the BCAS and sham groups were injected with saline. After the last injection, we examined cognitive function and exploration behavior using the Morris water maze. Mice brains were then harvested for Western blot analyses.

RESULTS: The BCAS group, but not the BCAS + α-LA group, showed cognitive dysfunction in the Morris water maze. Apoptosis pathways involving poly (ADP-ribose) polymerase (PARP) cleavage, phosphorylated-mammalian target of rapamycin (p-mTOR), phosphorylated-3-phosphoinositide-dependent protein kinase-1, and phosphorylated-protein kinase B (p-AKT) were enhanced in the BCAS group than the α-LA group. The BCAS + α-LA group demonstrated less PARP cleavage and p-mTOR function than did the BCAS group. The activity of autophagy pathways involving LC3B was higher in the BCAS and BCAS + α-LA groups than the sham group, but there were no significant differences between the BCAS and BCAS + α-LA groups.

CONCLUSIONS: In the BCAS rodent model, cognitive dysfunction and apoptosis mediated by the phosphatase and tensin homolog/AKT/mTOR pathway were observed in the hippocampus. However, acting on the mTOR pathway, α-LA improved cognitive function and led to hippocampal cell survival. Thus, α-LA may be useful for treating VaD.

Key Words:
Vascular dementia, α-Lipoic acid, Morris water maze.

Introduction

Vascular dementia (VaD) is characterized by an acquired cognitive impairment that occurs due to cerebrovascular disease. Generally associated with cerebrovascular disorders such as stroke, the risk factors for VaD include hypertension, diabetes mellitus, metabolic disorders, and metabolic syndrome. Cerebrovascular disease decreases the blood supply to the brain and elevates oxidative stress in the brain tissue by sequentially eliciting hypoxia, anoxia, and inflammatory responses. Further, cerebrovascular disease results in the loss of autonomic control of blood vessels, functional impairment of capillaries, and damage in the white matter and hippocampus; these pathophysiological diagnoses eventually lead to VaD symptoms[1].

Dementia syndromes are characterized by progressive deterioration of cognitive function and are highly prevalent in senior citizens[14]. While VaD has the highest incidence after Alzheimer's disease among dementia syndromes, there are currently no known therapeutic pharmacological treatments. Clinical treatment for VaD focuses only on the use of various drugs to prevent further cognitive deterioration. Among these drugs, α-LA is used – in part – as a potent antioxidant; however, its efficacy and pharmacological mechanism are poorly understood[1,15,16]. The organosulfur compound α-lipoic acid (α-LA; Thioctacid®) was isolated in the 1950s and has since been used as an antioxidant[2,3]. As a fatty acid synthesized by

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the mitochondria, α-LA eliminates reactive oxygen species such as hydroxyl radicals (free radicals), promotes the generation of glutathione, and recycles antioxidants such as vitamins E and C, thus serving an important role in the body's antioxidant network[4,5]. Since α-LA is easily absorbed and can cross the blood-brain barrier (BBB), it is known to protect against neurodegeneration[6] and neuropathic pain[7,8]. However, although α-LA has been shown to exert clinically positive effects, the mechanisms by which it functions in the brain and nervous system are still unclear. Also, the mechanisms underlying the functions of α-LA in the bilateral common carotid artery stenosis (BCAS) rodent model of VaD are still unclear.

Materials and Methods

Animals and Experimental Ethics

We used 8-week-old C57/BL male mice. Mice were acclimatized to the controlled laboratory conditions (temperature = 21 ± 2°C, humidity = 40-60%, 12-h light/dark cycle) for 1 week prior to experimentation to minimize stress levels. Mice were categorized into one of three groups using the Latin square procedure based on body weight, as follows: the sham group (n = 9), the BCAS group (n = 7), or the BCAS + α-LA group (n = 7). All investigations were approved by the Experimental Animal Ethics Committee at Wonkwang University, Korea.

Procedures for Bilateral Common Carotid Artery Stenosis Surgery

General anesthesia was performed on acclimatized 9-week-old C57/BL male mice using 2.5% sevoflurane (Baxter International Inc., Deerfield, IL, USA), N_2O, and a 95% oxygen gas mixture (Hankook Special Gases Co., Ltd., Jeollabuk-do, South Korea). Following the disinfection of the surgical site with Potadine (Povidone solution; Ilsung Pharmaceutical Co., Ltd., Seoul, South Korea), an incision, approximately 1 cm in diameter, was made along the cervical midline, and the common carotid arteries were isolated from the surrounding tissues. Upon exposure of the common carotid arteries, a gold-plated stenosis coil with an inner diameter of 0.18 mm (Sawane Spring Co., Shizuoka Prefecture, Japan) was used to constrict the bilateral common carotid arteries, reducing the blood flow to the brain. The surgical incision was sutured using 6-0 silk suture thread (Iri Suture Co., Seoul, South Korea) and disinfected with Povidone Iodide (Green Pharmaceutical Co., Chungbuk, South Korea). Mice were raised in a controlled environment for 4 weeks postoperatively to establish an experimental animal model of VaD[11,12]. For the BCAS + α-LA group, α-LA was intraperitoneally injected (100 mg/kg) once daily for 4 weeks after BCAS surgery. To control for stress conditions in the BCAS + α-LA group, the BCAS and sham groups were injected with a similar volume of saline once daily for 4 weeks.

The Morris Water Maze Test

A tubular water tank (black, diameter = 60 cm, height = 47 cm) was used as a water maze apparatus. A video camera, equipped with a video tracking system, was placed on the ceiling above the water tank to monitor the mice (EthoVision Version 3; EthoVision Co., Madrid, Spain) and was subsequently used to analyze the movement of the mice on a computer. One trial was conducted in each cardinal quadrant of the tank; one session was defined as a total of 4 trials. In each trial, the mouse was placed in the water and required to locate a submerged platform. The cut-off time was set at a maximum of 60 s for the first trial. If the mouse failed to locate the platform within the cut-off time in a trial, the experimenter would move the mouse to the location of the platform. The mouse was allowed to stay on the platform for 15 s in every trial in order to induce spatial navigation learning. Further, given the physical exhaustion involved in swimming in the water tank, mice were allowed some recovery time between trials. Recovery time between trials was maintained at 20 min to minimize differences in the interval between trials among mice. Following three training sessions over 3 days, a probe test session was administered to assess memory consolidation and learning ability. In the probe test session, each mouse was required to locate the platform in one trial. In contrast to the training session, in the probe session, the start position was opposite to the location of the platform, and the platform was removed before the session began. The probe test session was conducted for 60 s, and the time it took the mouse to find and dwell at the original location of the platform was measured. By dividing the water tank into four quadrants, we measured the dwelling time in the virtual platform per quadrant and the frequency of crossing the virtual platform location.

After the probe test, a re-training session was administered for 2 days, during which the platform was moved to the opposite side, and the

mouse was allowed to locate the new platform position. During the re-training session, the initial water maze training process was repeated in the same manner, with the platform at a different location; following the re-training session, a re-probe test was administered accordingly.

Protein Expression Analysis

Following the decapitation of the mice and brain extraction, the brain tissue was microdissected into distinct parts on a cold glass plate and snap frozen in liquid nitrogen. Tissue samples were homogenized in a buffer solution (25 mM Tris, 1 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid, 1 mM Dithiothreitol, 0.1% Triton-X 100, pH 7.4) for protein extraction and stored on ice for 1 h. Tissue lysates were subsequently centrifuged at 12,000 × *g* at 4°C, and the supernatant was isolated and quantified using the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA) and a spectrophotometer (Molecular Devices, San Jose, CA, USA). After the protein quantification assay, tissue lysates were diluted in 5X sample buffer (Elpis Biotech Inc., Daejeon, Korea) and shaken at 95°C for 5 min. Protein samples were loaded and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed at 80 V for 2 h, and protein samples were subsequently transferred to a 0.45-μm polyvinylidene difluoride (PVDF; Millipore Co., Billerica, MA, USA) membrane. To prevent non-specific binding of primary antibodies to the PVDF membrane, protein samples were incubated in 5% skim milk for 1 h at 24°C and washed in 0.05% Tris-Buffered Saline and Tween 20 (TBST) for 10 min three times. Primary antibodies were PSD95 (1:1000) α-phospho-AKT (thr308, D25E6; 1:1000), α-phospho-PTEN (ser380; 1:1000), α-phospho-PDK1 (ser241, C49H2; 1:1000), α-phospho-forkhead box O3 (FoxO3a) (ser253, D18H8; 1:1000), α-FoxO3a (D19A7; 1:1000), α-phospho-mTOR (ser2448; 1:1000), α-phospho-Raptor (ser792; 1:1000), α-Sequestosome-1 (SQSTM1)/p62 (1:1000), α-LC3B (1:1000), α-poly (ADP-ribose) polymerase-1/2 (PARP1/2) (1:1000), α-actin (1:1000), and α-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000). Protein samples were incubated with primary antibodies overnight at 4°C and washed twice in TBST for 10 min. Samples were then incubated with secondary antibody, α-rabbit immunoglobulin G (1:5000), for 1 h at room temperature and washed in TBST four times. Detection was performed using the FluorChem Systems (ProteinSimple, San Jose, CA, USA) and the enhanced chemiluminescence reagent (Millipore Co., Billerica, MA, USA).

Reagents

The α-phospho-AKT (thr308, D25E6), α-phospho-PTEN (ser380), α-phospho-PDK1 (ser241, C49H2), α-phospho-FoxO3a (ser253, D18H8), α-FoxO3a (D19A7), α-phospho-mTOR (ser2448), α-phospho-Raptor (ser792), α-SQSTM1/p62, α-LC3B, α-PARP1/2, and α-rabbit immunoglobulin G antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The α-actin and α-GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). We purchased the PVDF membranes and enhanced chemiluminescence kits from Millipore Co. (Billerica, MA, USA). The α-LA was a product of Bukwang Pharm Co. Ltd (Dongjak-gu, Seoul, South Korea).

Statistical Analysis

Statistical data and graphs were generated via performing analyses of variance (ANOVAs) using GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, USA). Values obtained from behavioral experiments are expressed as the mean ± the standard error; all other values are expressed as the mean ± the standard deviation. The significance of the differences between groups was determined by using Fisher's Least Significant Difference (LSD) post-hoc analyses (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

Results

Treatment With α-LA Improves BCAS-Induced Cognitive Dysfunction

The Morris water maze test was administered to determine the effects of α-LA on cognitive function recovery in the BCAS animal model. The mean time taken to locate the platform (escape latency) on day 1 was approximately 23.6 s (sham = 19.2 ± 3.8 s; BCAS = 25.6 ± 4.9 s; BCAS + α-LA = 26.0 ± 6.9, Figure 1A). The escape latency on day 1 was similar among all groups. Longer escape latencies was observed on the 3 days of the training (sham = 10.0 ± 1.5 s; BCAS = 23.1 ± 5.1 s; BCAS + α-LA = 11.6 ± 1.8, *p*-value = 0.004, Figure 1A) and 2 day of reverse training sessions in the BCAS group than the sham group (sham = 8.0 ± 0.8 s; BCAS = 12.2 ± 1.9 s; BCAS

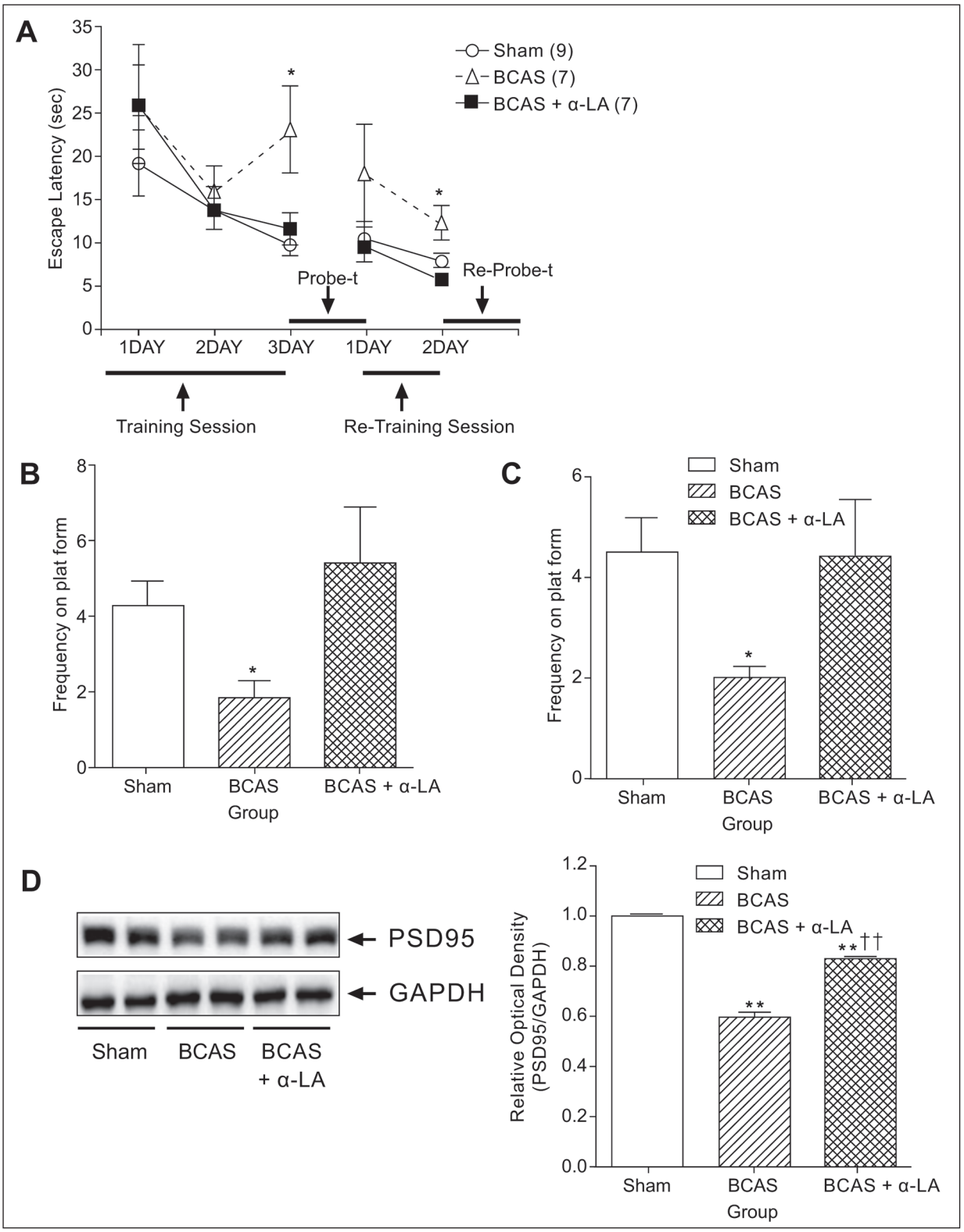

Figure 1. Effects of α-LA on BCAS-induced memory impairments in the Morris water maze task during the re-training and re-probe sessions. **A,** The learning curve in the water maze test. **B,** Number of accurate platform location crossings during the probe test and **(C)** re-probe-test. **D,** Expression of PSD95 in the hippocampus. The numbers in parentheses are the number of animals. A, B, C: data represent the mean ± the standard error. D: data represent the mean ± the standard deviation. *$p < 0.05$, **$p < 0.01$ *vs.* the sham group; $^{\dagger\dagger}p < 0.01$ *vs.* the BCAS group by ANOVA (post hoc tests performed with Fisher's LSD).

+ α-LA = 5.6 ± 0.8, *p*-value = 0.02 Figure 1A). However, the escape latency of the BCAS + α-LA and sham groups demonstrated similar results.

Consistently, based on our findings from the probe and re-probe tests, the learning accuracy of the BCAS group was significantly lower, and the learning accuracy of the BCAS + α-LA group was similar to that of the sham group (Figure 1B, C).

The protein expression of PSD95, a marker of hippocampal synaptic connection strength, was examined. Western blot analyses demonstrated that PSD95 expression levels were lower in the BCAS group than they were in the sham group, while the PSD95 expression levels in the BCAS + α-LA group were higher than were those in the BCAS group (Figure 1D).

Activation of the PTEN/AKT/mTOR Signaling Pathway Induces Apoptosis

We observed impaired cognitive function and decreased PSD95 protein expression in the hippocampus following BCAS. To determine the associations among decreased protein expression, oxidative stress, and neuronal damage in the central nervous system in our animal model of VaD, the status of the PTEN/AKT/mTOR signaling pathway was analyzed using Western blotting. We found that the expression level of p-mTOR and PARP was increased in BCAS group. But, in the BCAS + α-LA group, the expression level of phosphorylated (p)-PTEN was reduced while the expression levels of p-AKT, and p-FoxO3a were elevated in the BCAS + α-LA group (Figure 2A, B). In addition, in the BCAS + α-LA group, the expression level of p-PDK1 was elevated and the expression levels of p-mTOR and p-Raptor and PARP cleavage were reduced (Figure 2A, B).

Induction of Autophagy Occurs in the BCAS Model

The above results confirmed that the α-LA-associated reduction in PARP cleavage involved in apoptosis. The PTEN/AKT/mTOR pathway is also known to be associated with autophagy. The loss of PTEN function leads to the activation of mTOR, and the decrease in mTOR serves as an autophagy negative regulator that brings about increases in autophagy[9,10,13]. As shown in Figure 2, a reduction in PTEN and p-mTOR was observed in the BCAS + α-LA group. To establish whether these results are related to autophagy, the expression of the autophagosome markers LC3B and p62 was analyzed. We found that the LC3B expression level tends to increase in the BCAS and BCAS + α-LA groups than it was in the sham group but not significant. Moreover, while the p62 expression level was comparable between the sham and BCAS groups, it was higher in the both BCAS group and BCAS + α-LA group than the sham group. BCAS + α-LA group was higher in the BCAS group (Figure 3).

Discussion

We observed that, compared to the spatial navigation learning in the sham group, the spatial navigation learning in the BCAS group was impaired over the 3-day training session. In contrast, the escape latency of the BCAS + α-LA group was comparable to that of the sham group. These results confirm that treatment with α-LA attenuated the impairments in learning that were observed in the BCAS group. Similar findings were observed in the probe test and re-probe test. Comparative analysis of the strength of learning and/or memory consolidation showed that chronic cerebrovascular hypoperfusion led to the observed impaired cognitive function. These impairments were recovered by administration of α-LA.

In the present study, our quantification of PSD95 expression, a hippocampal marker of synaptic connection strength, demonstrated a 40% reduction in the protein expression level in the BCAS group and a 20% reduction in the BCAS + α-LA group when compared to the expression level in the sham group (Figure 1D). These findings suggest that BCAS results in neurochemical alterations that are associated with strengthening the neural network, while treatment with α-LA, an antioxidant, alleviates these alterations.

We also found the activation of the PTEN/AKT/mTOR signaling pathway by oxidative stress in an animal model of VaD. Dephosphorylation of PTEN causes phosphorylation of the downstream effectors PDK1 and AKT (thr308), which in turn activate mTOR complex 1[17]. Decreased expression of p-PTEN and increased expression of p-PDK1 and p-AKT were observed in the hippocampus of the BCAS group, and this was accompanied by the co-activation of p-mTOR (Figure 2). Further, we detected an elevation in the number of PARP cleavage fragments, which is an indicator of neuronal apoptosis[18], in BCAS group. In contrast, since treatment with the antioxidant α-LA produced reductions in the activa-

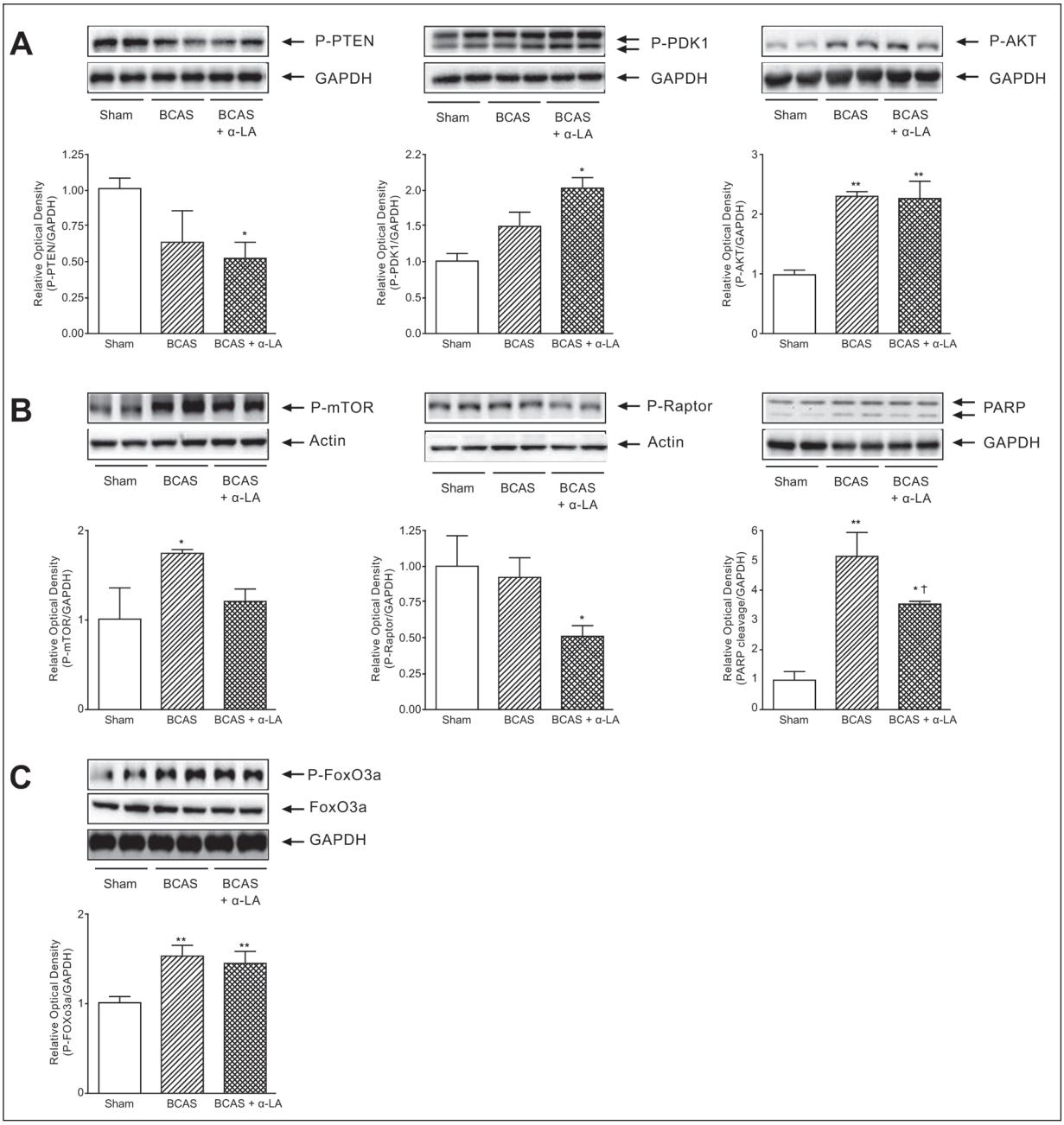

Figure 2. Expression of p-PTEN, p-PDK1, p-AKT, p-Raptor, p-mTOR, p-FoxO3a, FoxO3a, and PARP in the hippocampus. Graphic representation of the relative density of protein levels in the hippocampus. All experiments were performed in triplicate. Data represent the mean ± the standard deviation. *$p < 0.05$, **$p < 0.01$ *vs.* the sham group by ANOVA (post-hoc tests performed with Fisher's LSD).

tion levels of p-mTOR and p-Raptor and in PARP cleavage in the BCAS + α-LA group, neuronal apoptosis was likely suppressed, although these reductions were insufficient to restore the neuronal viability to the same level that was observed in the sham group (Figure 2). Here, we measured p-FoxO3a expression, which is associated with the PTEN/AKT/mTOR pathway. The expression of FoxO3 is regulated by stress conditions such as DNA damage, malnutrition, and hypoxia. It is also known to play important roles in cell proliferation, metabolism, stress responses and mechanisms. FoxO3a is activated by oxidative stress[19], and PTEN/AKT is involved in its regulation[20].

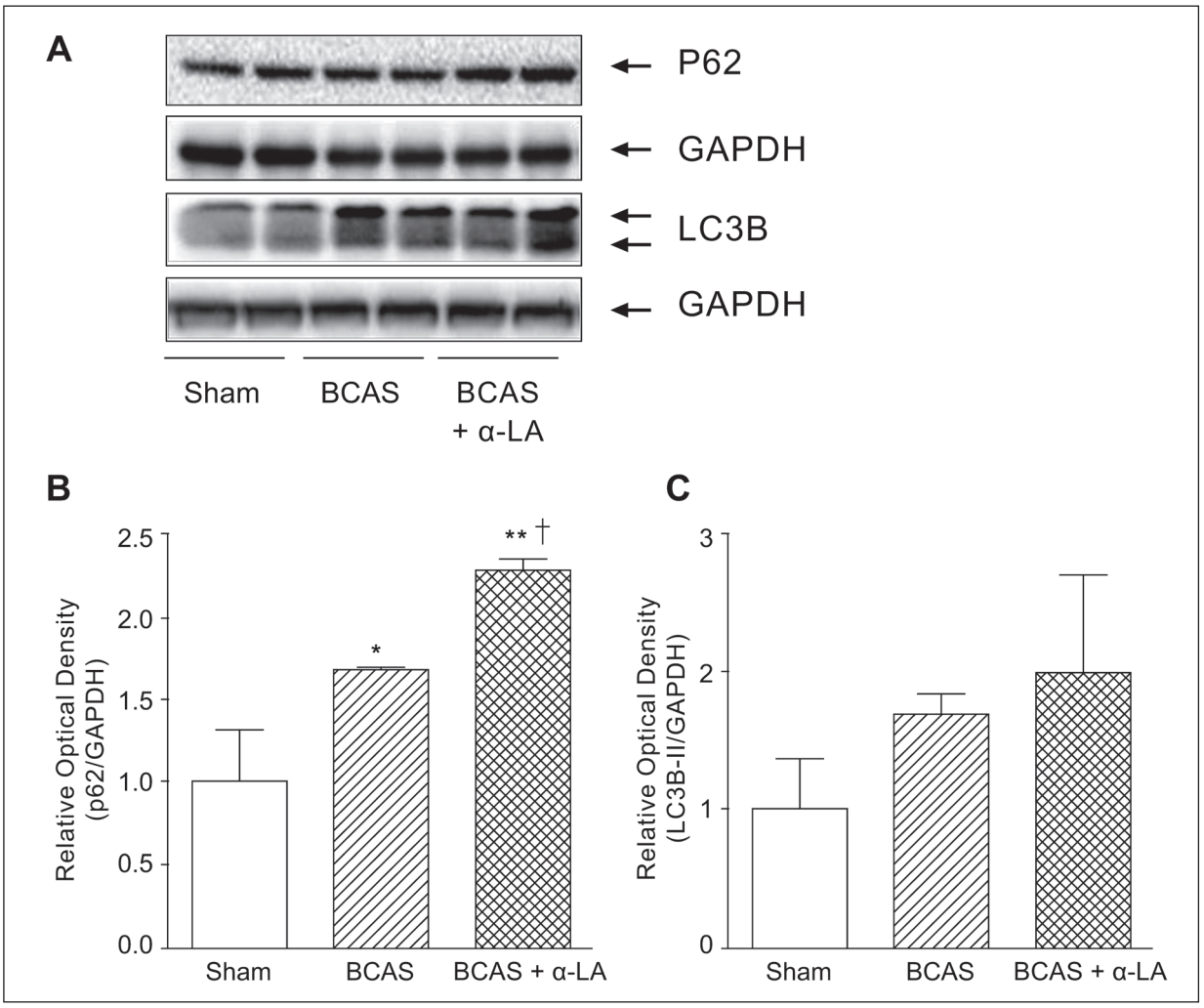

Figure 3. Expression of p62 and LC3B in the hippocampus. Graphic representation of the relative density of protein levels in the hippocampus. All experiments were performed in triplicate. Data represent the mean ± the standard deviation. *$p < 0.05$, **$p < 0.01$ *vs.* the sham group; $^{\dagger}p < 0.05$ *vs.* the BCAS group by ANOVA (post-hoc tests performed with Fisher's LSD).

The activation of AKT leads to lipid phosphorylation of FoxO3a, inhibiting the expression of pro-apoptotic genes[21,22]. We demonstrated that, in the BCAS group, p-PTEN expression was reduced than sham group, while the expression of p-AKT and FoxO3a, which are downstream of PTEN/AKT, was elevated. Similar findings were observed in the α-LA-treated group (Figure 2). Research shows that the PTEN/AKT/mTOR signaling pathway induces autophagy as well as apoptosis[9,10]. Thus, we examined the expression of LC3B and p62, markers of autophagy activation. LC3B and p62, which are expressed in the initial stages of the autophagy process, bind with damaged mitochondria and organelles that are to undergo autophagy. Although autophagy is generally known as an arbitrarily occurring catabolic process, some researches[23,24] suggest that a number of specific organelles are preferentially broken down and that p62 binds with the autophagosome after selectively binding to LC3B. In the current work, the expression levels of LC3B were higher in the BCAS and BCAS + α-LA groups than they were in the sham group; however, no significant differences were found between the BCAS and BCAS + α-LA groups. The elevation in p62 expression was greater in the BCAS + α-LA group than it was in the BCAS group (Figure 3). Scholars[23,25,26] indicate that p62 increases ubiquitinated protein aggregation and protects cell viability with autophagy substrate. In contrast, silencing p62 can activate autophagy,

as evidenced by the amount of autophagosomes that have been identified in multiple carcinoma cell lines[27]. Thus, p62 is cell-dependent and a deficiency in p62 leads to cell death in almost all cell lines. In our study, we found to enhance autophagy through α-LA-induced elevations in p62 levels and reductions in p-mTOR expression than BCAS group but no definite difference in the LC3B expression level was identified between the BCAS and BCAS + α-LA groups. We cautiously suggest that α-LA may lead to normal fluctuations in autophagy rather than to dysregulated autophagy.

Conclusions

We indicated that impaired cognitive function, similar to that observed in VaD, was induced by BCAS and that this impairment was attenuated by α-LA treatment. In this BCAS model, apoptosis was induced through the PTEN/AKT/mTOR pathway and autophagy in the hippocampus. Our findings demonstrate that α-LA exerts its neuroprotective effects in VaD by inhibiting mTOR activity and PARP cleavage and elevating p-PDK1 activity, thereby suppressing neuronal apoptosis. Therefore, we believe that α-LA has the potential to serve as a treatment for VaD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Declaration of Funding Interests

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