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Full Paper

Combined citicoline and docosahexaenoic acid treatment improves cognitive dysfunction following transient brain ischemia

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ABSTRACT

Phospholipids are structural components of cellular membranes that play important roles as precursors for various signaling pathways in modulating neuronal membrane function and maintenance of the intracellular environment. Phosphatidylcholine (PtdCho) is the most abundant cellular phospholipid. Citicoline and docosahexaenoic acid (DHA) are essential intermediates in the synthesis of PtdCho. Both PtdCho intermediates have independently shown neuroprotective effects in cerebral ischemia, but their combined effect is unknown. This study aimed to investigate the combined effect of oral citicoline and DHA treatment on improvement of cognitive deficits following cerebral ischemia using a 20-min bilateral common carotid artery occlusion (BCCAO) mouse model. BCCAO ischemic mice were treated for a total of 11 days with a combination of citicoline (40 mg/kg body weight/day) and DHA (300 mg/kg body weight/day) or each alone. Combined citicoline and DHA synergistically and significantly improved learning and memory ability of ischemic mice compared with either alone. Further, citicoline and DHA treatment significantly prevented neuronal cell death, and slightly increased DHA-containing PtdCho in the hippocampus, albeit not significantly. Taken together, these findings suggest that combined citicoline and DHA treatment may have synergistic benefits for partially improving memory deficits following transient brain ischemia.

1. Introduction

Cerebral ischemia is a leading cause of long-term disability that is associated with a high incidence of motor and cognitive dysfunction.[1] Approximately 23% of ischemic strokes are caused by transient ischemic attacks (TIA),[2] thus safe and effective therapy at the subacute phase after TIA is necessary to prevent consecutive brain damage.

Citicoline is a mononucleotide composed of cytosine, pyrophosphate, and choline, and an essential intermediate in the synthesis of phosphatidylcholine (PtdCho). Citicoline shows neuroprotective effects in several animal models of central nervous system injury, including cerebral ischemia.[3–5] Meanwhile in humans, many clinical stroke trials have shown that citicoline is the only drug with neuroprotective benefit. Consistently, pooled analysis has revealed an effect of oral citicoline in patients with moderate-to-severe acute ischemic stroke, however its effect appears limited. Recently, a large randomized controlled trial study (namely ICTUS) found similar global recovery between citicoline and placebo groups,[6] indicating that developing combinations of agents that show synergistic benefit with citicoline is required to enhance its limited effect in brain ischemia.

Post-ischemic progression and neuronal injury are regulated by multiple mechanisms, such as excitatory neurotransmitter accumulation, calcium overload, and free radical and fatty acid generation. In addition, citicoline acts at several levels of the ischemic cascade to repair the brain by stabilizing membrane phospholipids.[7] During brain ischemia, membrane phospholipids are degraded to fatty acids, which are key molecules of the ischemic

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cascade.[8] Indeed, Lopes-Coviella et al., reported that citicoline (500 mg/kg body weight) administered orally to rats for 90 days increased the amount of PtdCho in the frontoparietal cortex by approximately 30%.[9]

Docosahexaenoic acid (22:6, DHA) is an essential polyunsaturated fatty acid that is enriched in neural membranes and especially found in PtdCho, phosphatidylethanolamine, and other brain membrane phosphatides. DHA is required for brain development and plays an important role in brain function.[10] Deficiency of DHA in the brain has been implicated in cognitive decline. In rats, DHA is reported to improve spatial cognitive deficits induced by transient forebrain ischemia,[11] suggesting that DHA in brain tissue may act as an antioxidant against free radical generation.[12] In gerbils, oral administration of DHA (300 mg/kg/day) for 28 days increases brain PtdCho levels by 22% as well as synaptic protein levels.[13]

Both precursors of PtdCho, citicoline and DHA, have a neuroprotective and enhancing effect on PtdCho synthesis in the brain, but their combined effect is still unknown. Hence, the aim of our study was to investigate whether administering citicoline and DHA has a synergistic effect on improving cognitive dysfunction, neuronal cell death, and molecular changes in phospholipids in the hippocampus of mice subjected to bilateral common carotid artery occlusion (BCCAO).

2. Materials and methods

2.1. Animals

Ten-week-old male C57BL/6 mice were purchased from Crea Japan (Yokohama, Japan) and group housed at a constant temperature (23 ± 1 °C) and humidity (55 ± 5%) with lights on from 9:00 to 21:00 and *ad libitum* access to food and water. After arrival, mice were habituated to the environment for 1 week, and fed a laboratory commercial eicosapentaenoic acid (EPA)- and DHA-deficient diet prepared by deleting fish oil from standard diet (F-1; Funabashi Farm, Chiba, Japan). All experimental procedures using animals were approved by the Committee on Animal Experiments of Tohoku University.

2.2. Experimental design, preparation of ischemic model, and dietary treatment

After acclimation, mice were divided into six groups: sham-control, sham-combination (DHA 300 mg/kg body weight + citicoline 40 mg/kg body weight), BCCAO-control, BCCAO-DHA (300 mg/kg body weight), BCCAO-citicoline (40 mg/kg body weight), and BCCAO-combination (DHA 300 mg/kg body weight + citicoline 40 mg/kg body weight).

BCCAO mice were prepared by surgery, as described previously.[14] Briefly, mice were anesthetized with 4% halothane and maintained with 2% halothane (Takeda Chemical Industries Ltd, Osaka, Japan), then bilateral common carotid arteries were occluded for 20 min via a lateral neck incision. One day after surgery, mice were orally treated daily by gavage at a constant volume of 10 mL/kg for 11 consecutive days. To reach final treatment doses, citicoline was dissolved in distilled water and DHA suspended in 0.5% (w/v) methyl cellulose, with both reagents mixed well before every use. Citicoline was provided by Kyowa Hakko Bio (Cognizin®; Tokyo, Japan), DHA was purchased as DHA-rich oil (DHA™-S; Martek Biosciences Co., Columbia, MD, USA) containing 35.7% DHA. Control mice were treated with distilled water as the vehicle. Animals were subjected to behavioral tests from least to most stressful: Y-maze task test at 8 days after surgery, novel object recognition at 8–9 days after surgery, and step-through passive avoidance task at 10–11 days after surgery.[15] During behavioral tasks, animals were administered each reagent after the task. Oral administration was performed by 11 days after surgery in all groups. At 12 days after surgery, mice were sacrificed and brain tissue dissected for further analysis.

2.3. Y-maze task

Spontaneous alteration in the Y-maze task was used to determine willingness to explore new environments and spatial reference memory. The Y-maze task was performed as described previously[16] (see also Supplementary Material). Alternations were defined as entries into all three independent arms on consecutive choices. Percentage of alternations was calculated as actual alternations/maximum alternations × 100, with the number of actual alternations reflecting continuous time of entry to each of three arms. Moreover, number of maximum alternations was the total number of arms entered minus two. Total number of arms entered during the session was also determined.

2.4. Novel object recognition task

The novel object recognition task was performed as described previously[17] but with small modifications (see Supplementary Material). The novel object recognition task consisted of training and retention trials. Discrimination of spatial novelty was assessed by comparing differences between exploratory contact of novel and familiar objects. Total number of contacts with both objects was recorded to adjust for differences in total exploration contact.

2.5. Step-through passive avoidance task

The step-through passive avoidance task was performed as described previously[17] (see also Supplementary Material). The step-through passive avoidance task consisted of training and retention trials. Step-through latency was recorded at 300 s to assess the level of retention.

2.6. Histopathological analysis

Neuronal cell death was evaluated by histopathological analysis with propidium iodide (PI) at 12 days after ischemia. Histopathological analysis was performed as described previously[17] (see also Supplementary Material). Total number of viable cells from the left and right hippocampus of each animal was calculated as an average of two randomly chosen 500 × 500 μm areas from three different slices. Cell viability was expressed as a percentage of average number of viable cells from control animals. Position of the hippocampal CA1 region was identified by Paxinos and Franklin (2001).[18]

2.7. Western blotting analysis

After decapitation of mice, the brain was rapidly removed and tissue from the dorsal hippocampus dissected, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis. Western blot analyses were performed as described previously[16] (see also Supplementary Material). Two antibodies were used: anti-Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-α (1:5,000)[19] and anti-β-tubulin (1:10,000) (Sigma–Aldrich, St. Louis, MO, USA).

2.8. PtdCho analysis in hippocampus

After decapitation of mice, the brain was rapidly removed and tissue from the dorsal hippocampus dissected, immediately frozen

in liquid nitrogen, and stored at −80 °C until analysis. For liquid chromatography–Fourier transform type mass spectrometry (LC–FTMS) analysis, tissue samples from three mice were used, based on the results of the novel object recognition task. Frozen hippocampus was crushed frozen 3-times (1,500 rpm, 10 sec) with a metal corn and Multi-beads Shocker (Yasui Kikai, Osaka, Japan). The sample was then mixed with methanol containing PtdCho (16:0, D31-18:1; Avanti Polar Lipids, Alabaster, AL, USA) as the internal qualitative standard, and crushed frozen once (1,500 rpm, 10 min). After adding dichloromethane, the mixture was crushed frozen once again (1,500 rpm, 10 min), and then centrifuged (9,100 g, 5 min). The upper phase was resuspended in 10-times volume of acetone and stored at −80 °C until use for LC–FTMS. LC–FTMS analysis was performed by Chemicals Evaluation and Research Institute (Tokyo, Japan) using a UFLC XR system (Shimadzu, Tokyo, Japan) coupled with a LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA). Lipids were eluted from a column (L-column 2 ODS Metal-free, 2 × 150 mm, Particle size 3 μm; Chemicals Evaluation and Research Institute) heated at 40 °C using mobile phase A (19.6 mM ammonium formate) and mobile phase B (acetone) at a flow rate of 0.2 μL/min. Column effluent was directed into the electrospray source. Full MS scan range was 300–1,200 m/z, and spectra were acquired automatically in data-dependent top N5 scan mode (positive). Peak processing was performed using Mzmine 2 (http://mzmine.sourceforge.net/, ver. 2). Detected peaks had intensity >5,000, and were identified by their lipid class and carbon:double carbon number by m/z score and MS/MS spectra. Peak data are shown as area normalized by area of each internal control.

2.9. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using IBM SPSS Statistics version 25.0 (SPSS Inc., Chicago, IL, USA). Significant differences were determined by one-way analysis of variance (ANOVA) followed by multi-group comparisons between each group, then sham-control versus the other groups using Dunnett's multiple comparison test. In addition significant differences were determined by one-way analysis of variance (ANOVA) followed by multi-group comparisons between each BCCAO group, and BCCAO-control group versus BCCAO-treatment groups using Dunnett's multiple comparison test. For the novel object recognition test, Student's *t*-test was used to compare novel and familiar objects. Two-tailed *P* values < 0.05 were considered statistically significant in all analyses.

3. Results

3.1. Combined citicoline and DHA treatment improves impaired memory-related behavior in BCCAO mice

We determined whether orally-administrated citicoline (40 mg/kg) and DHA (300 mg/kg), either alone or in combination, improve memory impairment observed in BCCAO mice. Dosage of citicoline and DHA was determined according to a preliminary trial (data not shown), ineffective dosage of citicoline and DHA were chosen to clarify their combined effect. The Y-maze task assesses the willingness of mice to explore new environments and spatial working memory. Percentage of alterations in behavior significantly decreased in BCCAO-control mice compared with sham-control mice ($P < 0.05$; Fig. 1A) without any change in total number of arm entries (Fig. 1B). Treatment with DHA (300 mg/kg) to BCCAO mice significantly improved alteration behavior compared with BCCAO-control mice ($P < 0.05$; Fig. 1A) without any change in total number of arm entries (Fig. 1B). Percentage of alteration behavior with combined citicoline and DHA was higher compared with BCCAO-control mice, but no significant difference was observed ($P = 0.117$; Fig. 1A).

The novel object recognition task assesses recognition memory. In the training session, no difference was observed in discrimination index using the same object in all animal groups (Fig. 1C). After a 24 h retention interval between the training and test session, sham-control mice showed a significantly higher discrimination index for the novel object ($P < 0.01$; Fig. 1D). In contrast, BCCAO-control mice failed to discriminate between familiar and novel objects (Fig. 1D). This indicates that BCCAO mice show typical memory impairment behavior. Under the same conditions, BCCAO mice treated with a combination of citicoline and DHA showed a significantly higher discrimination index for the novel object ($P < 0.01$; Fig. 1D). While with DHA or citicoline treatment alone, mice failed to discriminate between familiar and novel objects (Fig. 1D).

The fear-conditioned passive avoidance task assesses contextual memory. In the conditioning training session, no significant differences were observed in any group in latency time to enter a dark compartment (Fig. 1E). However, latency time to entering a dark compartment was significantly decreased by 24 h after electric foot shock in BCCAO-control mice compared with sham-control mice ($P < 0.05$; Fig. 1F). This indicates that BCCAO mice show typical memory impairment behavior. Treatment with a combination of citicoline and DHA significantly restored the reduction in latency time compared with BCCAO-control mice ($P < 0.05$; Fig. 1F). Treatment with DHA or citicoline alone significantly decreased latency time compared with sham-control mice ($P < 0.01$), and showed no restoration of reduction in latency time compared with BCCAO-control mice (Fig. 1F).

These findings indicate that a combination of citicoline and DHA treatment has a synergistic ability to improve contextual memory and recognition memory deficits in BCCAO ischemia-induced mice, but not sham-treated mice.

3.2. Delayed neuronal cell death following BCCAO ischemia is prevented by combined citicoline and DHA treatment

Following staining with PI, viable cells were counted in the hippocampal CA1 region at 12 days after BCCAO ischemia to examine neuroprotective effects. As previously reported,[17] cell viability significantly decreased in BCCAO-control mice compared with sham-control mice ($P < 0.01$; Fig. 2A,B). Combined citicoline and DHA treatment prevented neuronal cell death induced by BCCAO ischemia in the hippocampal CA1 region ($P < 0.01$; Fig. 2A,B). Although treatment with either DHA or citicoline alone significantly decreased cell viability compared with sham-control mice ($P < 0.01$), with no restoration of delayed neuronal cell death compared with BCCAO-control mice (Fig. 2A,B). These findings indicate that a combination of citicoline and DHA treatment has a neuroprotective effect on BCCAO ischemia.

3.3. DHA treatment restores reduced CaMKIIα expression in the hippocampal CA1 region of BCCAO mice

CaMKIIα is predominantly expressed in neurons,[20] and CaMKIIα (CaMKIIα) was significantly decreased in the hippocampal CA1 region after BCCAO ischemia ($P < 0.01$; Fig. 3A,B). In Fig. 3A, CaMKIIα was detected at 50 kDa using antibodies against CaMKIIα, but non-specific bands were also observed at 60 kDa, which appear to be CaMKIIβ, γ and/or δ, because CaMKIIα shows high homology with these three proteins. Unexpectedly, treatment with a combination of citicoline and DHA showed an increase in CaMKIIα, albeit no significant difference, compared with BCCAO-control mice

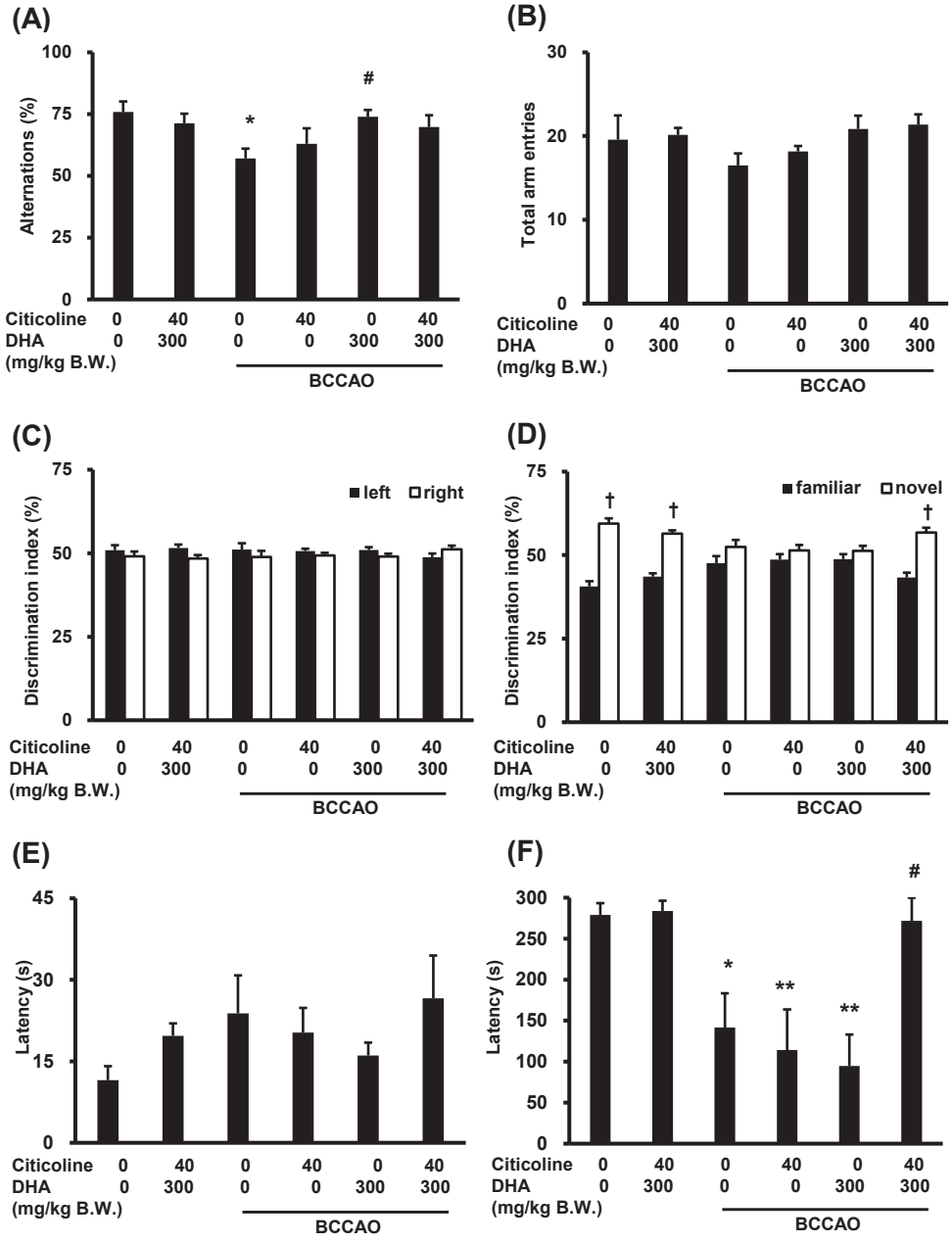

Fig. 1. Effect of combined citicoline and docosahexaenoic acid treatment on impaired memory-related behavior following BCCAO ischemia. Alternation (A) or total arm entry (B) in the Y-maze task. Number of objects in the trial session (C) or trial session (D) of the novel object recognition task. Latency time in the training session (E) or test session (F) of the passive avoidance task. All data represent mean ± S.E. ($n = 6–8$ per group). $^{*}P < 0.05$, vs. sham-control; $^{**}P < 0.01$, vs. sham-control; $^{\#}P < 0.05$, vs. BCCAO-control; $^{\dagger}P < 0.01$ vs. familiar group. BCCAO, bilateral common carotid artery occlusion.

($P = 0.119$; Fig. 3A,B). Decreased CaMKIIα protein following BCCAO ischemia was significantly restored by DHA ($P < 0.05$; Fig. 3A,B).

3.4. Molecular changes of PtdCho in the hippocampus of BCCAO mice by combined citicoline and DHA treatment

It remains unclear whether citicoline and DHA treatment in combination or alone can alter molecular species of PtdCho in the hippocampus of BCCAO mice. Approximately 1,000 peaks were detected, and 28 PtdCho species distributed in all groups were identified by LC–FTMS analysis (Supplementary Material). Miyawaki et al., reported 10 PtdCho species abundant in rat hippocampus, specifically, deacyl-16:0/16:0, deacyl-16:0/16:1, deacyl-16:0/18:1, deacyl-18:0/18:1, deacyl-16:0/20:4, deacyl-18:0/20:4, deacyl-18:1/20:4, deacyl-16:0/22:6, deacyl-18:0/22:6, and deacyl-18:1/22:6.[21] We assumed that PtdCho with ≥4 double carbons is arachidonic acid (AA) (20:4) containing PtdCho, and PtdCho with ≥6 double carbons is DHA containing PtdCho. Fig. 4 shows the ratio of DHA-containing PtdCho (DHA-PtdCho) and AA-containing PtdCho (AA-PtdCho). There were no significant differences observed following ischemia, but as expected the ratio of DHA-PtdCho/AA-PtdCho was higher in the hippocampus after combined citicoline and DHA treatment (BCCAO-control, 0.53 ± 0.016; BCCAO-combination, 0.56 ± 0.006), interestingly over DHA alone (BCCAO-DHA; 0.54 ± 0.010) (Fig. 4; mean ± S.E.). In addition, combined citicoline and DHA treatment significantly increased the

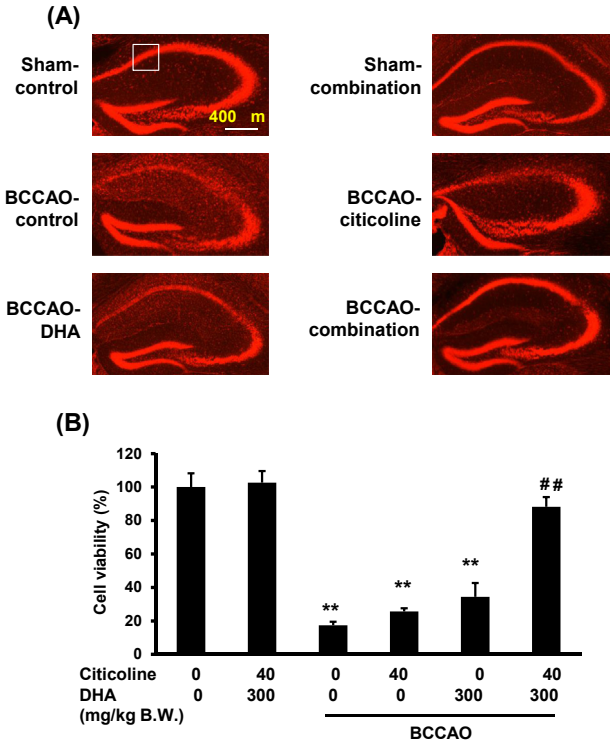

Fig. 2. Neuroprotective effect of combined citicoline and docosahexaenoic acid treatment against delayed neuronal cell death after transient BCCAO ischemia. (A) Representative histological sections of the hippocampus. (B) Cell viability is expressed as percentage of average number of viable cells from control mice. All data in cell viability represent mean ± S.E. ($n = 4$–6 per group). $^{**}P < 0.01$, vs. sham-control; $^{\#\#}P < 0.01$, vs. BCCAO-control. BCCAO, bilateral common carotid artery occlusion.

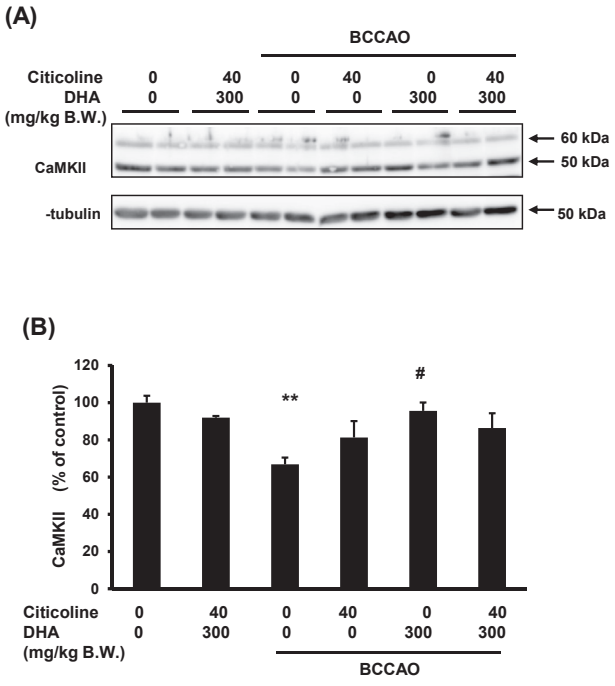

Fig. 3. Effect on CaMKII protein levels after transient BCCAO ischemia. (A) Representative western blots of CaMKIIα and β-tubulin in the hippocampal CA1 region. (B) Quantitative protein analysis of CaMKIIα. Blots of β-tubulin indicate equal protein loading in each lane. All quantitative protein analysis data represent mean ± S.E. ($n = 6$ per group). $^{**}P < 0.01$, vs. sham-control; $^{\#}P < 0.05$, vs. BCCAO-control. BCCAO, bilateral common carotid artery occlusion.

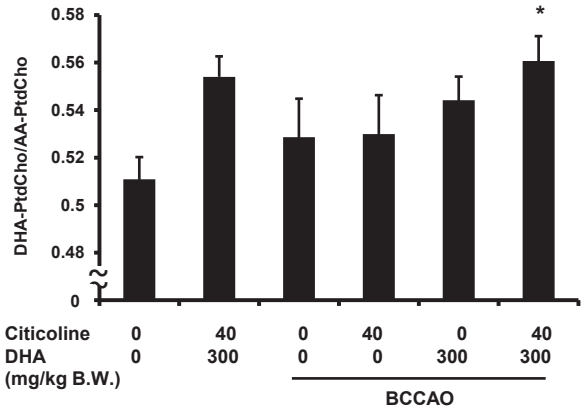

Fig. 4. Molecular changes in PtdCho by treatment of citicoline, DHA, and their combination. Peak area ratio of docosahexaenoic acid (DHA)-phosphatidylcholine (PtdCho) and arachidonic acid (AA)-PtdCho in the hippocampus. $^{*}P < 0.05$, vs. sham-control. All data represent mean ± S.E. ($n = 3$ per group).

ratio of DHA-PtdCho/AA-PtdCho in BCCAO-mice compared with sham-control mice ($P < 0.05$).

4. Discussion

Although we have shown that a combination of citicoline and DHA treatment partially improves cognitive dysfunction in BCCAO mice, its mechanism remains unclear. PtdCho reportedly decreases due to activated membrane-bound phospholipase A_2 in the brain, which results from cerebral ischemic damage.[8] Arachidonic acid is released as free fatty acid from degraded phospholipids by phospholipase A_2, and accelerates inflammation due to eicosanoids generated via an AA cascade. Thus, DHA is expected to suppress hippocampal neuronal damage, and consequently be incorporated into tissue and competitively inhibit AA, along with its own antioxidant effect.[12] Citicoline is widely used as an ischemic protective agent, which stabilizes phospholipid metabolism.[22] Moreover, citicoline is demonstrated to inhibit activity of membrane-bound phospholipase A_2.[23] From these reports, citicoline and DHA are qualitatively and quantitatively effective treatments for cerebral ischemia though PtdCho metabolism, which decreases during ischemia. Considering mechanisms, it is tempting to speculate that citicoline promotes uptake of DHA into brain tissue and synergistically rescue BCCAO deficits.

We found that DHA-PtdCho in the hippocampus may synergistically increase after combined citicoline and DHA treatment, even though we did not identify a statistically significant difference (Fig. 4). This suggests that citicoline can support incorporation of DHA into brain tissue. Hippocampal PtdCho has recently been reported to decrease once by 24 h after ischemia and recover after 7 days, suggesting that change in amount of molecular PtdCho species is an apoptotic signal.[21] Thus, it is possible that any change in amount of PtdCho has already recovered by 12 days after ischemia, which is the current sampling period. In addition, BCCAO mice showed delayed neuronal cell death in the hippocampus (mainly CA1 region), with a few affected neurons in other regions,[24,25] whereas we used the entire hippocampus for LC–FTMS analysis. Consideration of appropriate sampling time and brain region may be needed to confirm these results.

A synergistic effect of citicoline and DHA treatment on the benefit of learning and memory ability was not observed in the Y maze task for working memory. For increased understanding on combined citicoline and DHA treatment, we might identify appropriate conditions, especially for intake dose, and change our

focus to the prefrontal cortex using a different ischemia model. For example, in this study, BCCAO caused neuronal damage, particularly to the hippocampal CA1 region (Fig. 2A). In animal studies, working memory was originally used to explain hippocampal function, however working memory is also controlled by the prefrontal cortex.[26] DHA treatment to rat ischemia reduced infarct volume mainly in cortical, and neuronal cell death, by modulating the neuroinflammatory response.[27] The effect of DHA to cortex might in part account for improvement of working memory. Alternatively, recovery of CaMKII levels and neuroprotection might be contingent upon a balance in degree of ischemia and efficacy of treatment. Yamamoto et al., reported that nobiletin partially protected neurons but failed to rescue CaMKII levels, because BCCAO duration might affect consistent recovery of CaMKII levels and neuroprotection.[14] It may be needed further understandings on the dynamic behavior of the recovery of CaMKII levels and neuroprotection caused by BCCAO.

In conclusion, our results suggest that citicoline and DHA exert synergistic activity for improving learning and memory ability by preventing neuronal cell death following transient BCCAO ischemia. Further, this effect may be related to PtdCho species, especially DHA-containing PtdCho. More studies in another animal model or clinically, need to be performed in the future to determine the ability of DHA to enhance neuroprotection of citicoline.

Conflict of interest

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Appendix A. Supplementary data

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References

1. Das RR, Seshadri S, Beiser AS, et al. Prevalence and correlates of silent cerebral infarcts in the Framingham offspring study. *Stroke J Cereb Circ*. 2008;39(11):2929–2935.
2. Rothwell PM, Warlow CP. Timing of TIAs preceding stroke: time window for prevention is very short. *Neurology*. 2005;64(5):817–820.
3. Kakihana M, Fukuda N, Suno, et al. Effects of CDP-choline on neurologic deficits and cerebral glucose metabolism in a rat model of cerebral ischemia. *Stroke J Cereb Circ*. 1988;19(2):217–222.
4. Aronowski J, Strong R, Grotta JC. Citicoline for treatment of experimental focal ischemia: histologic and behavioral outcome. *Neurol Res*. 1996;18(6):570–574.
5. Andersen M, Overgaard K, Meden P, et al. Effects of citicoline combined with thrombolytic therapy in a rat embolic stroke model. *Stroke J Cereb Circ*. 1999;30(7):1464–1471.
6. Davalos A, Alvarez-Sabin J, Castillo J, et al. Citicoline in the treatment of acute ischaemic stroke: an international, randomised, multicentre, placebo-controlled study (ICTUS trial). *Lancet (London, England)*. 2012;380(9839):349–357.
7. Overgaard K. The effects of citicoline on acute ischemic stroke: a review. *J Stroke Cerebrovasc Dis Off J Natl Stroke Assoc*. 2014;23(7):1764–1769.
8. Koizumi S, Yamamoto S, Hayasaka T, et al. Imaging mass spectrometry revealed the production of lyso-phosphatidylcholine in the injured ischemic rat brain. *Neuroscience*. 2010;168(1):219–225.
9. Lopez-Coviella I, Agut J, Savci V, et al. Evidence that 5'-cytidinediphosphocholine can affect brain phospholipid composition by increasing choline and cytidine plasma levels. *J Neurochem*. 1995;65(2):889–894.
10. Lauritzen L, Hansen HS, Jorgensen MH, et al. The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Prog Lipid Res*. 2001;40(1–2):1–94.
11. Okada M, Amamoto T, Tomonaga M, et al. The chronic administration of docosahexaenoic acid reduces the spatial cognitive deficit following transient forebrain ischemia in rats. *Neuroscience*. 1996;71(1):17–25.
12. Horrocks LA, Farooqui AA. Docosahexaenoic acid in the diet: its importance in maintenance and restoration of neural membrane function. *Prostaglandins Leukot Essent Fatty Acids*. 2004;70(4):361–372.
13. Wurtman RJ, Ulus IH, Cansev M, et al. Synaptic proteins and phospholipids are increased in gerbil brain by administering uridine plus docosahexaenoic acid orally. *Brain Res*. 2006;1088(1):83–92.
14. Yamamoto Y, Shioda N, Han F, et al. Nobiletin improves brain ischemia-induced learning and memory deficits through stimulation of CaMKII and CREB phosphorylation. *Brain Res*. 2009;1295:218–229.
15. McIlwain KL, Merriweather MY, Yuva-Paylor LA, et al. The use of behavioral test batteries: effects of training history. *Physiol Behav*. 2001;73(5):705–717.
16. Moriguchi S, Yabuki Y, Fukunaga K. Reduced calcium/calmodulin-dependent protein kinase II activity in the hippocampus is associated with impaired cognitive function in MPTP-treated mice. *J Neurochem*. 2012;120(4):541–551.
17. Yabuki Y, Fukunaga K. Oral administration of glutathione improves memory deficits following transient brain ischemia by reducing brain oxidative stress. *Neuroscience*. 2013;250:394–407.
18. Paxinos G, Franklin KB. *The mouse brain in stereotaxic coordinates*. 2nd ed. Academic Press; 2000.
19. Fukunaga K, Goto S, Miyamoto E. Immunohistochemical localization of Ca2+/calmodulin-dependent protein kinase II in rat brain and various tissues. *J Neurochem*. 1988;51(4):1070–1078.
20. Erondu NE, Kennedy MB. Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain. *J Neurosci Off J Soc Neurosci*. 1985;5(12):3270–3277.
21. Miyawaki S, Imai H, Hayasaka T, et al. Imaging mass spectrometry detects dynamic changes of phosphatidylcholine in rat hippocampal CA1 after transient global ischemia. *Neuroscience*. 2016;322:66–77.
22. Secades JJ. Citicoline: pharmacological and clinical review, 2016 update. *Rev Neurol*. 2016;63(S03):S1–S73.
23. Arrigoni E, Averet N, Cohadon F. Effects of CDP-choline on phospholipase A2 and cholinephosphotransferase activities following a cryogenic brain injury in the rabbit. *Biochem Pharmacol*. 1987;36(21):3697–3700.
24. Sheng H, Laskowitz DT, Pearlstein RD, et al. Characterization of a recovery global cerebral ischemia model in the mouse. *J Neurosci Methods*. 1999;88(1):103–109.
25. Olsson T, Wieloch T, Smith ML. Brain damage in a mouse model of global cerebral ischemia. Effect of NMDA receptor blockade. *Brain Res*. 2003;982(2):260–269.
26. Funahashi S. Working memory in the prefrontal cortex. *Brain Sci*. 2017;7(5):49–71.
27. Eady TN, Belayev L, Khoutorova L, et al. Docosahexaenoic acid signaling modulates cell survival in experimental ischemic stroke penumbra and initiates long-term repair in young and aged rats. *PLoS One*. 2012;7(10), e46151.