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Antioxidative and neuroprotective effects of ascidiacea-derived plasmalogen in a mouse stroke model

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Abstract

Reactive oxygen species promote the oxidation of cellar DNA, lipids, and proteins, and are overproduced during cerebral ischemia and reperfusion. Ascidiacea-derived plasmalogen (aPlas) has both an anti-oxidative molecular structure of a vinyl ether bond at the *sn*-1 position and the bioactivity of DHA and EPA. In the present study, we examined the possible therapeutic potential of aPlas for 30 min of transient middle cerebral artery occlusion (tMCAO) mice. Vehicle or aPlas (10 mg/kg/day) were administrated intraperitoneally for two weeks before tMCAO. The mice were continuously treated with vehicle or aPlas, and were sacrificed after 5 days of reperfusion. aPlas treatment showed a neuroprotective effect on tMCAO mice. aPlas significantly decreased infarct volume (vehicle: 90.9 ± 21.3 mm^3, aPlas: 64.5 ± 23.4 mm^3, $p<0.05$) and reactive oxygen metabolite levels in serum (d-ROM test; vehicle: 182.0 ± 15.5 CARR U, aPlas: 163.7 ± 12.7 CARR U, $p<0.05$). Moreover, aPlas protected the brain against oxidative damage to DNA/RNA and lipid peroxidation, suppressed the activation of microglia M1, and promoted the regeneration of neurons. These results suggest that aPlas may be a safe therapeutic candidate against ischemic stroke.

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Abbreviations: AD, Alzheimer's disease; aPlas, ascidiacea-derived purified plasmalogen; CARR U, Carratelli units; CML, nε-carboxymethyl lysine; DAB, diaminobenzidine; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Iba-1, ionized calcium binding adapter protein 1; NeuN, neuronal nuclei; PBS, phosphate-buffered saline; Plas, plasmalogen; pGAP-43, phosphorylated growth associated protein 43; PFA, paraformaldehyde; ROS, reactive oxygen species; tMCAO, transient middle cerebral artery occlusion; 4-HNE, 4-hydroxynonenal; 8-OHdG, 8-hydroxydeoxyguanosine.

Key words: anti-oxidative effect, ischemic stroke, neuroinflammation, oxidative stress, plasmalogen, reactive oxygen species.

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Introduction

Recent studies have consistently demonstrated that early tissue reperfusion confines the ischemic core, leads to a reduction in infarct volume, and restores neurological functions. However, such recanalization may also result in tissue damage known as reperfusion injury (Abe et al., 1988; Molina and Alvarez-Sabin, 2009). Reactive oxygen species (ROS) promote oxidation of cellar DNA, lipids, and proteins, and are overproduced during cerebral ischemia and reperfusion (Morimoto et al., 1996; Abe K, 2000; Zhang et al., 2004; Chan PH, 2016). We previously reported that suppression of oxidative stress by using a free radical scavenger edaravone and a dietary supplement was effective for neuroprotection and improvement of prognosis in ischemic stroke mice (Abe et al., 1988; Zhang et al., 2000; Zhang et al., 2004; Yamashita et al., 2009; Takamiya et al., 2012; Jiao et al., 2018; Shang et al., 2018). However, a more effective and convenient treatment of ischemic stroke is required.

Plasmalogen (Plas) is a special class of glycerophospholipids that have a vinyl ether bond at the *sn*-1 position of the glycerol moiety, and is present in all mammalian tissues, especially in the heart and brain. The vinyl ether bond at the *sn*-1 of the glycerol moiety is targeted by a variety of oxidants, including ROS. Therefore, Plas can act as an antioxidant and protect cells from oxidative stress (Farooqui and Horrocks, 2001; Braverman and Moser, 2012; Hossain et al., 2016; Fujino et al., 2017). In addition, Plas has a polyunsaturated fatty acid such as docosahexaenoic acid (DHA; C22:6) or eicosapentaenoic acid (EPA; C20:5) at the *sn*-2 position (Su et al., 2019), which may result in an additional anti-oxidative effect.

In the present study, therefore, we examined the possible therapeutic potential of ascidiacea-derived purified Plas (aPlas) for a mouse stroke model.

Materials and Methods

Animals and pretreatment

All animal experiments were performed in compliance with the guidelines following approval by the Animal Care and Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (approval #OKU-2020480), and conducted in accordance with ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines) and the Okayama University guidelines on the Care and Use of Laboratory Animals. Adult male C57BL/6J mice (20-25 g, 7 weeks old; n=38) were purchased from Japan SLC Inc. (Shizuoka, Japan). After resting for one week, mice were randomly divided into two experimental groups: the vehicle group and the aPlas-pretreated group. Each group was intraperitoneally injected with O.D.O., which is a medium-chain triglyceride with a non-greasy feel (The Nisshin OilliO Group, Ltd.; 500 µl/day, n=19) or aPlas (provided by Sunsho Pharmaceutical Co., Ltd. Shizuoka, Japan; 10 mg/kg/day, n=19) for two weeks.

Focal cerebral ischemia

Focal cerebral ischemia was introduced into mice by transient middle cerebral artery occlusion (tMCAO) according to our previous reports (Abe et al., 1992; Yamashita et al., 2017). Briefly, mice were anesthetized with a nitrous oxide: oxygen: isoflurane mixture (69%:30%:1%, respectively) through an inhalation mask. The left common carotid artery was exposed and a 7-0 nylon thread with a silicone-coated tip was inserted into the right middle cerebral artery (MCA) while body temperature was maintained at 37 ± 0.3°C using a heat bed (BWT-100; Bio Research Center). After 30 min of tMCAO, the silicone-coated

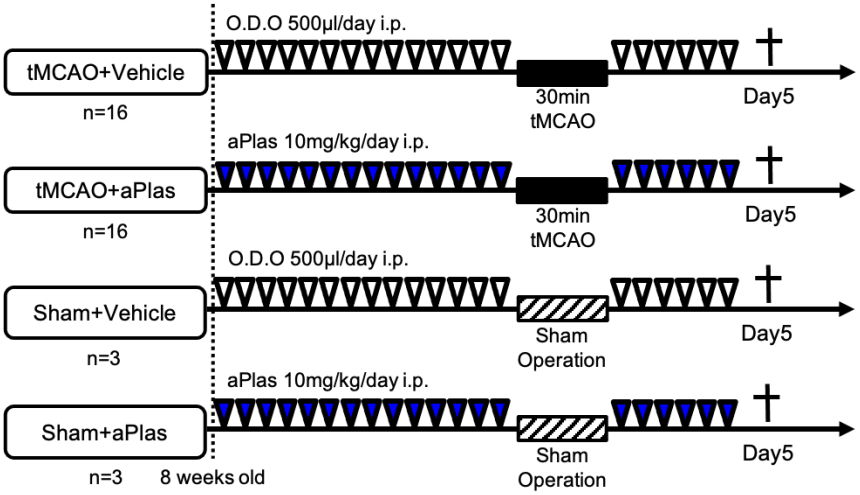

Fig. 1 Schematic diagram of the experimental procedure. Arrowheads indicate the intraperitoneal injection of O.D.O. or aPlas.

thread was pulled out to restore blood flow (reperfusion). The incision was then closed and the animals recovered and were allowed free access to water and food at ambient temperature. Three sham control mice were selected from the two experimental groups and received a sham cervical operation but without inserting the thread.

Assessment of neurological deficit scores after tMCAO

The mice were evaluated for behavioral changes at 1 and 5 days after reperfusion. Bederson's scale scores with minor modifications were applied in this study (Bederson et al., 1986). Scoring criteria were as follows: 0, no observable neurologic deficits; 1, failure to extend the left forepaw; 2, circling to the left side; 3, falling to the left side; 4, no spontaneous walking.

A corner test was also carried out to detect impairment of sensorimotor function, which is correlated with ischemic lesion volume and the neurological score (Zhang et al., 2002). Briefly, the edges of two boards were attached at an angle of 30 degrees, and a mouse was placed between the two angled boards facing the corner. The non-ischemic mouse could turn either left or right, but the ischemic mouse preferentially turns toward the non-impaired side. This test was repeated 10 times for each mouse and the number of left turns (the impaired side) was recorded.

Infarct volumes

The mice were sacrificed at 5 days after tMCAO. Each mouse was anesthetized by intraperitoneal injection of pentobarbital (20 mg/kg), and then perfused with chilled PBS, followed by 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer. After postfixation in the same fixative for 12 h at 4°C, whole brains were cut into 50-μm-thick sections with a vibrating blade microtome (VT1000S; Leica, Wetzlar, Germany). Brain sections were stained with cresyl violet as Nissl staining to measure the infarct area using a standard computer-assisted image analysis technique. The infarct volume of each brain was calculated by adding infarct areas of five serial brain slices, at a 0.6 mm interval each, between 1.0 mm anterior and 1.5 mm posterior to the bregma.

Oxidative serum status

To measure two types of oxidative status in mice serum, the d-ROM test and the OXY-adsorbent test (Diacron International, Italy) were used according to the

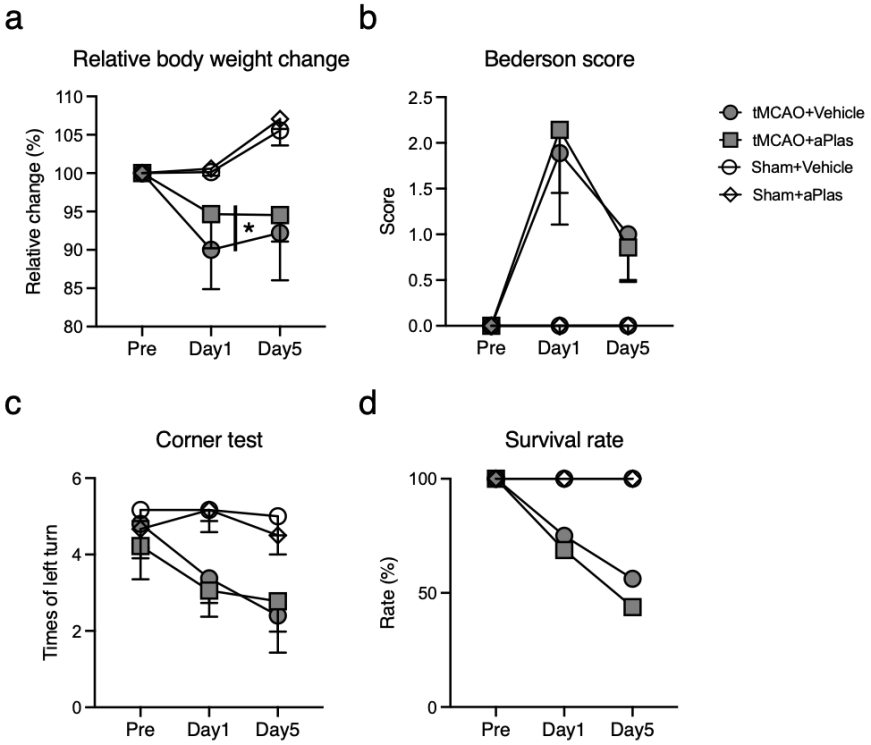

Fig. 2 Clinical scores of mice at 1 or 5 days after tMCAO. (a) Relative body weight change, (b) Bederson score, (c) corner test, and (d) survival rate were compered between tMCAO + vehicle group and tMCAO + aPlas group by using the Mann-Whitney's U test. Values of $p < 0.05$ were considered to be significantly different.

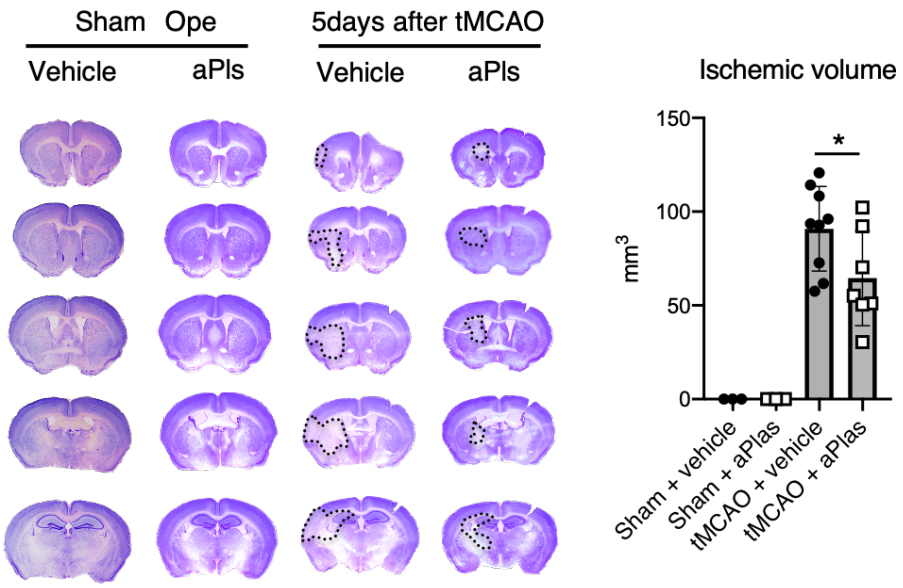

Fig. 3 Nissl staining was performed at 5 days after tMCAO or sham operation in the vehicle and aPlas group mice brains, and ischemic infarct volume was calculated. Note that aPlas significantly decreased the volume of cerebral ischemic infarction compared to the vehicle group (* $p<0.05$).

manufacturer's instruction and our previous reports (Kusaki et al., 2017; Taira et al., 2020). The d-ROM test measures hydroperoxides, which are derived from the oxidation of fatty acids, proteins and nucleic acids and can promote cell death. The spectrophotometer (Free Radical Elective Evaluator, Diacron International, Grosseto, Italy) showed reactive oxygen metabolite levels that are expressed as arbitrary 'Carratelli units' (CARR U), with 1 CARR U corresponding to 0.08 mg per 100 mL H_2O_2. The OXY-adsorbent test quantifies the ability of serum to oppose the massive oxidative action of hypochlorous acid through different antioxidant compounds present in serum (Beaulieu et al., 2020). The spectrophotometer automatically calculated antioxidant capacity as the level of HClO erased by the serum (mmol HClO/mL).

Immunohistochemistry

For immunohistochemistry, free-floating sections were incubated in 0.3% hydrogen peroxide/methanol for 20 min to prevent endogenous peroxidase activity. After washing three times in PBS, brain sections were blocked in 5% bovine serum albumin for 2 h then incubated at 4°C overnight with the following primary antibodies. All antibodies used in this study were from commercial sources: mouse anti-8OHdG antibody (1:100; JaICA; MOG-020P); mouse anti-CML antibody (1:100; CAC, AGE-M01); mouse anti-4HNE antibody (1:50; JaICA, MHN-020P). The sections were washed in PBS and incubated with biotinylated secondary antibodies (Vector Laboratories) diluted at 1:500 for 2 h. The sections were then incubated with the ABC Elite complex (Vector Laboratories), and visualized with diaminobenzidine (DAB) substrate dissolved in PBS.

For double immunofluorescent analysis, sections were incubated with primary antibodies overnight and then incubated with secondary antibodies Alexa Fluor™ (1:500; Invitrogen) for 2 h at room temperature. The primary antibodies were as follows: rabbit anti-Iba1 antibody (1:500, Wako; 019-19741), mouse anti-CD16/CD32 antibody (1:1000, BD Biosciences; 553141), rabbit anti-NeuN antibody (1:1000, abcam; ab104225), mouse anti phosphorylated GAP-43 S96 antibody (1:250, Wako, 010-25401).

Statistical analysis

All data are presented as mean ± S.D. in the text. Statistical analyses were performed using statistical software (SPSS 22.0.0.0; IBM, Armonk, NY, USA). After checking for normality, we performed the Mann-Whitney's U test to compare changes in relative body weight, Bederson score, corner test score, survival rate, and ischemic volume between tMCAO + vehicle group and tMCAO + aPlas group. Other statistical analyses were performed with the Kraskal-Wallis test to compare the d-ROM test, the OXY-adsorbent test, and the number of positive cells counted (8OHdG, CML, 4HNE) among the two sham groups and the two tMCAO groups. Values of $P < 0.05$ were considered to be significantly different.

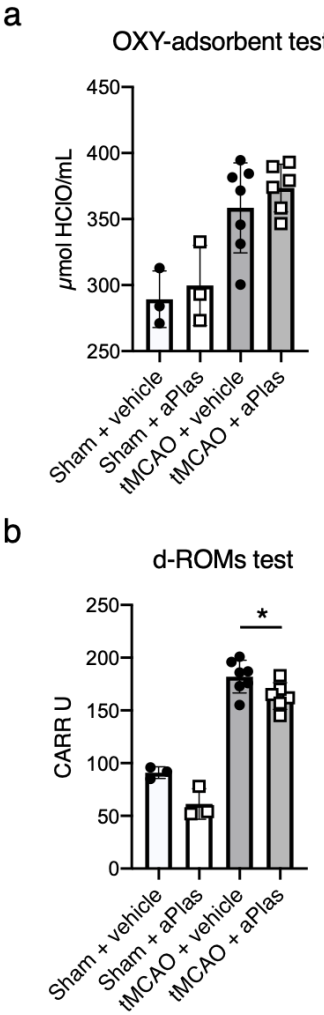

Fig. 4 Serum levels of reactive oxygen metabolites (d-ROMs) and antioxidant capacity (OXY-adsorbent), showing that aPlas significantly inhibited reactive oxygen metabolite levels after tMCAO compared to the vehicle group (* $p<0.05$). There was no significant difference between the vehicle group and the aPlas group in the OXY-adsorbent test. infarction compared to the vehicle group (* $p<0.05$).

Results

Clinical scores and cerebral ischemic infarction volume

Although there were no significant differences in the Bederson score, the corner test, and survival rate at 1 or 5 days after tMCAO between the vehicle group and the aPlas treatment group (Fig. 2b-d), aPlas treatment significantly suppressed weight loss at 1 day after tMCAO (Fig. 2a, * $p<0.05$).

aPlas significantly decreased the volume of cerebral ischemic infarction compared to the vehicle group at 5 days after tMCAO (Fig. 3, vehicle: 90.9 ± 21.3 mm^3, aPlas: 64.5 ± 23.4 mm^3, * $p<0.05$).

Antioxidative activities and oxidative stress marker in serum

There was no significant difference between the vehicle group and the aPlas group after the sham (vehicle: 289.3 ± 21.4 μmol HCLO/mL, aPlas: 299.7 ± 30.2 μmol HCLO/mL) operation as well as tMCAO (vehicle: 358.5 ± 34. 0 μmol HCLO/mL, aPlas: 373.5 ± 18.1 μmol HCLO/mL) in the OXY-adsorbent test (Fig. 4a). On the other hand, aPlas significantly inhibited reactive oxygen metabolite levels after tMCAO compared to the vehicle group (vehicle: 182.0 ± 15.5 CARR U, aPlas: 163.7 ± 12.7 CARR U, * $p<0.05$) in the d-ROM test (Fig. 4b), although there was no significant difference between the two sham operation groups (vehicle: 91.0 ± 5.6 CARR U, aPlas: 61.3 ± 14.5 CARR U).

Oxidative stress marker in brain

8-OHdG was slightly labeled in the nuclei of both sham groups (vehicle: 25.7 ± 8.5 cells / mm^2, aPlas: 24.0 ± 9.6 cells / mm^2) (Fig. 5a upper left, 5b). The number of positive cells in the peri-infarct fields were strongly observed in the vehicle group at 5 days after tMCAO. However, aPlas treatment significantly decreased the number of positive cells (vehicle: 124.3 ± 13.8 cells / mm^2, aPlas: 103.7 ± 13.5 cells / mm^2, * $p<0.05$) (Fig. 5a upper right, 5b).

Correspondingly, lipid peroxidation marker 4-HNE was labeled slightly in the cytoplasm and cytomembrane of neural cells of both sham groups (vehicle: 22.3 ± 5.1 cells / mm^2, aPlas: 10.0 ± 3.0 cells / mm^2), and the number of positive cells was increased by tMCAO but was significantly suppressed by aPlas treatment (vehicle: 103.4 ± 18.2 cells / mm^2, aPlas: 81.1 ± 18.5 cells / mm^2, * $p<0.05$) (Fig. 5a lower, 5d).

There were no significant differences between CML-positive cells in the vehicle group and the aPlas treatment group (sham + vehicle: 12.3 ± 4.7 cells / mm^2, sham + aPlas: 9.7 ± 2,1 cells / mm^2, tMCAO + vehicle: 159.2 ± 24.1 cells / mm^2, tMCAO + aPlas: 143.6 ± 13.2 cells / mm^2) (Fig. 5a middle, 5c).

Anti-inflammatory effects and neural regeneration

Iba-1-positive cells in the peri-infarct fields were strongly observed in both vehicle and aPlas groups at 5 days after tMCAO. However, the expression of CD16/32 was attenuated in the aPlas groups not only after tMCAO but also after the sham operation (Fig. 6a).

Double immunofluorescent analysis showed that pGAP-43 was detected in the peri-infarct areas of both the vehicle and aPlas groups, although it was expressed more strongly in the aPlas group than in the vehicle group (Fig. 6b).

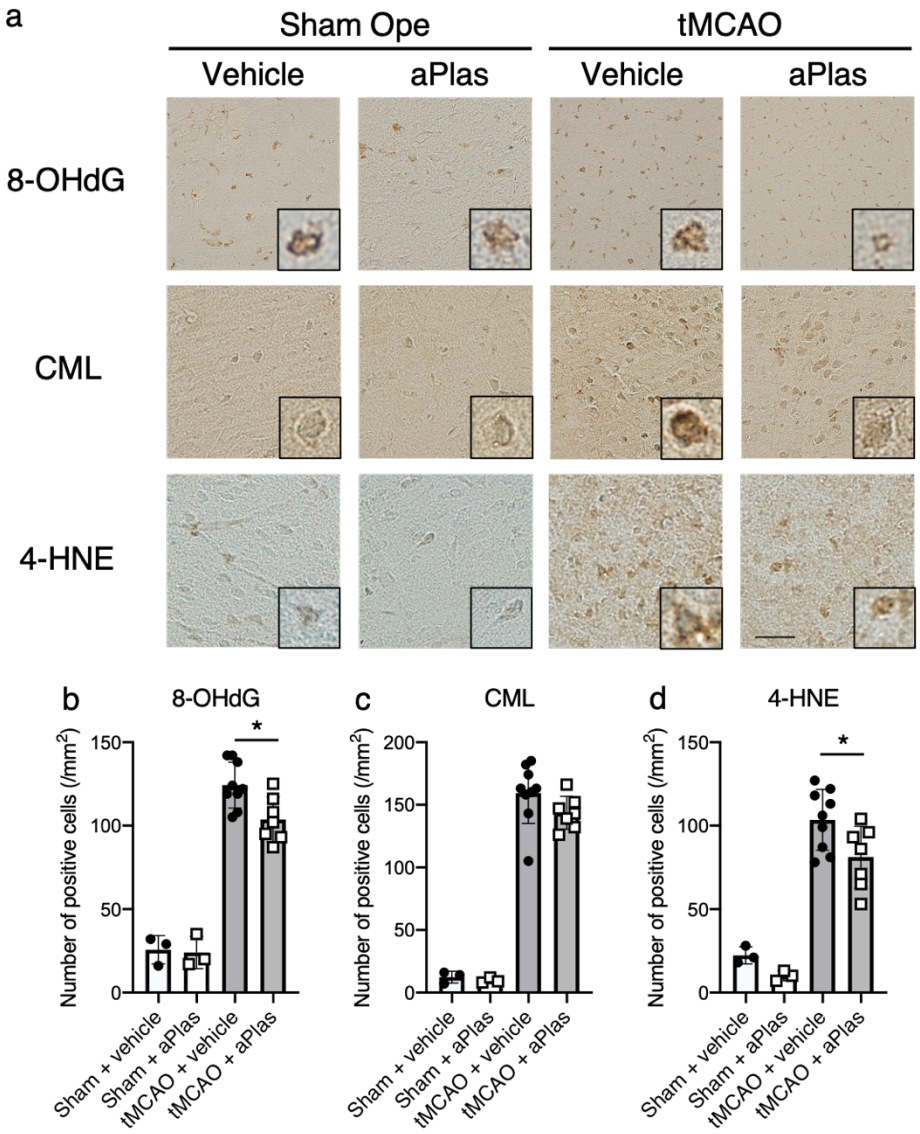

Fig. 5 Immunohistochemistry for 8-OHdG, CML, and 4-HNE at the peri-infarct area (a) and quantitative analyses of positive cells (b). The number of 8-OHdG- and 4-HNE-positive cells were increased by tMCAO and were significantly suppressed by aPlas treatment (* $p<0.05$). Scale bars: 100 μm.

Discussion

In the present study, we demonstrated that aPlas treatment had a neuroprotective effect on the focal cerebral ischemia model of mice. aPlas significantly decreased infarct volume (Fig. 3) and reactive oxygen metabolite levels in serum (Fig. 4b). Moreover, aPlas offered protection against oxidative damage to DNA/RNA and lipid peroxidation in the brain (Fig. 5), and suppressed microglia M1 activation and promoted regenerating neurons (Fig. 6).

Relative body weight loss at one day after tMCAO was suppressed by aPlas treatment when compared with the vehicle group (Fig. 2a). Plas are glycerophospholipids that have vinyl ether double bonds at the *sn*-1 position and have a polyunsaturated fatty acid such as DHA (C22:6) or EPA (C20:5) at the *sn*-2 position (Fujino et al., 2017; Su et al., 2019). Several studies in some cancer patients showed that DHA and/or EPA reduced weight loss (Whitehouse et al., 2001; van der Meji et al, 2010; Murphy et al., 2011; Weed et al., 2011; Pappalardo et al., 2015). Plas may play an important role in weight loss after tMCAO via the ATP-ubiquitin-dependent proteolytic pathway.

ROS become exaggerated during reperfusion to trigger oxidative damage to DNA, lipids, and proteins (Chan PH, 1996; Peters et al., 1998; Chan PH, 2001; Jung et al., 2010; Lakhan et al., 2009). Therefore, scavenging ROS and inhibiting oxidative stress are frequently focused as a medically treatable targets (Abe et al., 1995; Peters et al., 1998; Kusaki et al., 2017). In

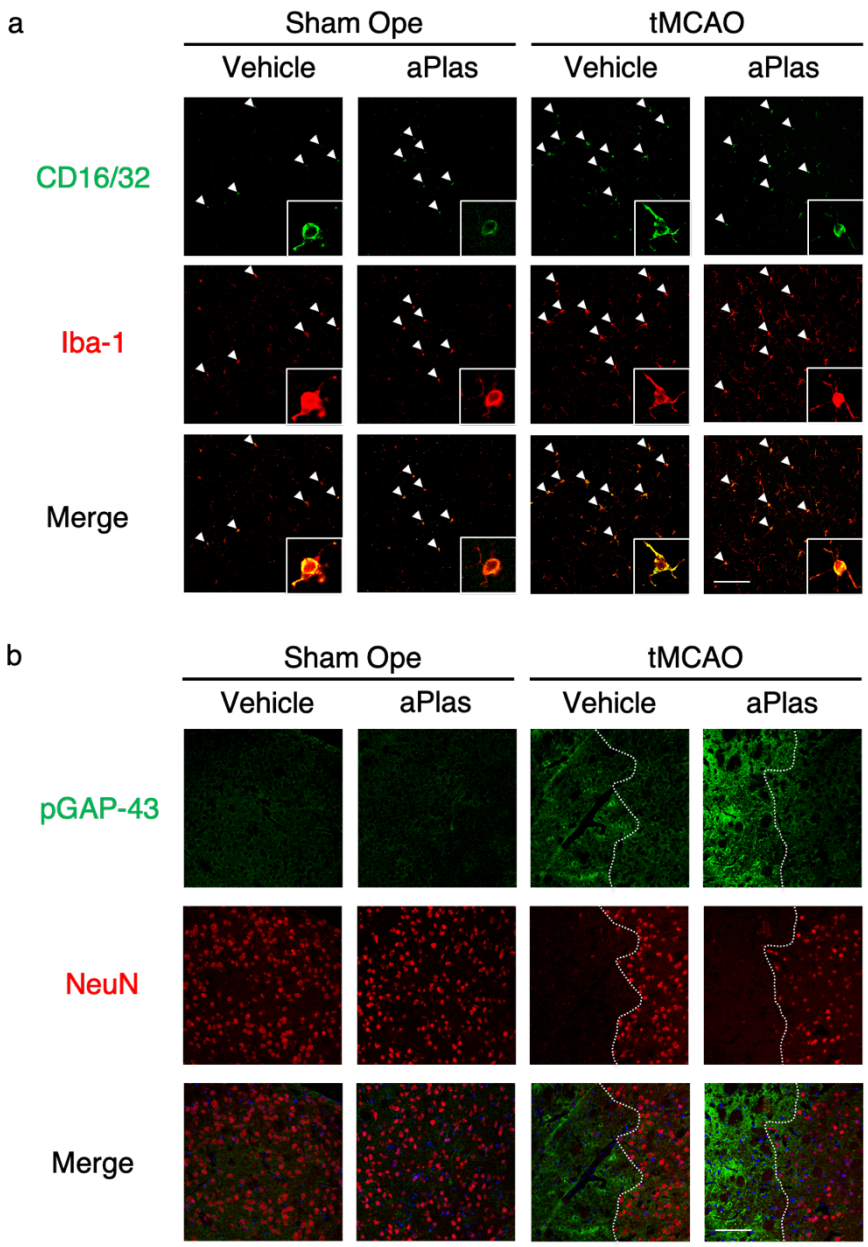

Fig. 6 Immunofluorescent analysis of (a) CD16/32 and Iba-1, and (b) pGAP-43 and NeuN in the peri-ischemic area at 5 days after tMCAO. Arrowheads indicate CD16/32 and Iba-1 double-positive cells. Scale bars: 100 µm.

the present study, we elucidated that aPlas suppressed reactive oxygen metabolite levels in serum after tMCAO (Fig. 4b), oxidative damage to RNA and DNA (8-OHdG, Fig. 5a, b), and lipid peroxidation (4-HNE, Fig. 5a, d). These mechanisms may be due to both the molecular structure of a vinyl ether bond at the *sn*-1 position and the bioactivity of DHA and EPA (Sosenko IR, 1995; Pratico et al., 2001).

Experimentally and clinically, strokes are followed by the sustained or excessive activation of M1 phenotype microglia to secondary damage (Kriz J, 2006; Hu et al., 2012; Wang et al., 2013). Although the activation of microglia and macrophages were observed in the peri-infarct fields at 5 days after tMCAO (Fig. 6a middle), aPlas altered the M1 phenotype microglia and macrophages which aggravate tissue damage by producing pro-inflammatory cytokines (Fig. 6a upper). Moreover, aPlas promoted the repair of damaged neurons (Fig. 6b). Serin 96 phosphorylation of neuronal growth-associated 43-kDa (pGAP-43) was specifically detected in growing and regenerating axons as the most frequent target of JNK signaling (Kawasaki et al., 2018; Che et al., 2018; Youssef et al., 2019). aPlas may induce a synergistic effect between the molecular structural feature of a vinyl ether bond at the *sn*-1 position and the bioactivity of DHA and EPA (Barber et al., 1999).

In conclusion, we successfully demonstrated the neuroprotective effects of aPlas on adult male mice at 5 days after tMCAO via a decrease in infarct volume, alleviating serum and cerebral oxidative stress, promoting the repair of damaged neurons, and attenuating neuroinflammation. The present study provides evidence of the potential of aPlas as a safe and therapeutic candidate against ischemic stroke.

Conflicts of interests

The authors disclose no potential conflicts of interest.

Acknowledgements

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