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Selective Toll-Like Receptor 4 Antagonists Prevent Acute Blood-Brain Barrier Disruption After Subarachnoid Hemorrhage in Mice

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Abstract

There are no direct evidences showing the linkage between Toll-like receptor 4 (TLR4) and blood-brain barrier (BBB) disruption after subarachnoid hemorrhage (SAH). The purpose of this study was to examine if selective blockage of TLR4 prevents BBB disruption after SAH in mice and if the TLR4 signaling involves mitogen-activated protein kinases (MAPKs). One hundred and fifty-one C57BL/6 male mice underwent sham or endovascular perforation SAH operation, randomly followed by an intracerebroventricular infusion of vehicle or two dosages (117 or 585 ng) of a selective TLR4 antagonist IAXO-102 at 30 min post-operation. The effects were evaluated by survival rates, neurological scores, and brain water content at 24–72 h and immunoglobulin G immunostaining and Western blotting at 24 h post-SAH. IAXO-102 significantly prevented post-SAH neurological impairments, brain edema, and BBB disruption, resulting in improved survival rates. IAXO-102 also significantly suppressed post-SAH activation of a major isoform of MAPK p46 c-Jun N-terminal kinase (JNK) and matrix metalloproteinase-9 as well as periostin induction and preserved tight junction protein zona occludens-1. Another selective TLR4 antagonist TAK-242, which has a different binding site from IAXO-102, also showed similar effects to IAXO-102. This study first provided the evidence that TLR4 signaling is involved in post-SAH acute BBB disruption and that the signaling is mediated at least partly by JNK activation. TLR4-targeted therapy may be promising to reduce post-SAH morbidities and mortalities.

Keywords Antagonist · Blood-brain barrier disruption · Neuroinflammation · Subarachnoid hemorrhage · Toll-like receptor 4

Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is a cerebrovascular disease with devastating consequences [1, 2]. Recent studies have suggested that early brain injury (EBI) is the primary determinant of poor outcomes after SAH [3]. Blood-brain barrier (BBB) disruption is one of the important pathological manifestations of EBI, and post-SAH BBB disruption allows inflammatory substances and plasma components to enter into the brain parenchyma, causing further aggravation of neuroinflammation and brain injuries [4]. Thus, treatment against BBB disruption may be an important strategy to improve outcomes after SAH, but the molecular mechanisms of post-SAH BBB disruption remain poorly understood.

Neuroinflammation is a well-recognized consequence of SAH and possibly implicated in EBI after SAH [5]. Of the Toll-like receptor (TLR) family members, TLR4 possibly plays a critical role in initiating inflammatory reactions after SAH, leading to EBI [6–9]. TLR4 signaling involves activation of nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs), both of which produce pro-inflammatory mediators such as interleukins (ILs), matrix metalloproteinase (MMP)-9, tenascin-C (TNC), and periostin [5, 10–14]. These pro-inflammatory mediators and MAPKs including c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) 1/2 have been reported to be involved in BBB disruption after experimental SAH [5, 11, 13, 15]. However, previous studies have exclusively focused on TLR4/NF-κB signaling pathway [9, 16, 17], and TLR4/MAPK signaling pathway has never been investigated as to post-SAH EBI including BBB disruption. In addition, selective TLR4 antagonists have never been tested for EBI after SAH. Thus, the aim of this study was to investigate if selective blockage of TLR4

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prevents BBB disruption after SAH in mice and if the TLR4 signaling involves MAPKs.

Materials and Methods

The Animal Ethics Review Committee of Mie University approved the study protocol, and all experiments were conducted in accordance with the institution's Guidelines for Animal Experiments.

Animal Model and Study Protocol

C57BL/6 male mice (weight, 25–30 g) were used to produce SAH models by endovascular perforation as previously described [18]. Briefly, each mouse was anesthetized by an intraperitoneal injection of tribromoethanol (250 μg/g body weight). After exposing the left common, external, and internal carotid arteries, a sharpened 4-0 monofilament nylon suture was advanced rostrally into the left internal carotid artery from the external carotid artery stump to perforate the bifurcation of anterior and middle cerebral arteries. Blood pressure and heart rate were noninvasively monitored during operation from the tail. Body temperature was kept constant at 37 °C during the operation. Sham-operated mice underwent identical procedures except that the suture was withdrawn without a puncture.

First, to study the effects of a TLR4 antagonist on post-SAH BBB disruption, 169 mice underwent endovascular perforation SAH ($n = 133$) or sham ($n = 36$) operation. At 30 min after operation, 151 surviving mice were randomly divided into five groups as follows: sham + phosphate-buffered saline (PBS; $n = 30$), sham + IAXO-102 585 ng ($n = 6$), SAH + PBS ($n = 56$), SAH + IAXO-102 117 ng ($n = 45$), and SAH + IAXO-102 585 ng ($n = 14$) groups. A selective TLR4 antagonist IAXO-102 (AdipoGen, San Diego, CA) or the vehicle was administered intracerebroventricularly. Mortality and neurological scores were evaluated at 24, 48, and 72 h, and SAH severity and brain water content were assessed at 24 and 72 h. BBB permeability and Western blotting were evaluated at 24 h post-operation (Fig. 1a; Online Resource, Table S1).

Next, to confirm the involvement of TLR4 in post-SAH BBB disruption, 25 mice underwent endovascular perforation SAH ($n = 18$) or sham ($n = 7$) operation. At 30 min post-operation, 24 surviving mice were randomly divided into three groups as follows: sham + TAK-242 72 ng ($n = 7$), SAH + PBS ($n = 10$), and SAH + TAK-242 72 ng ($n = 7$) groups. Another selective TLR4 antagonist TAK-242 (EMD Millipore, Billerica, MA) or the vehicle was administered intracerebroventricularly. Mortality, neurological scores, SAH severity, BBB permeability, and immunohistochemical staining were assessed at 24 h post-operation (Fig. 1b; Online Resource, Table S1).

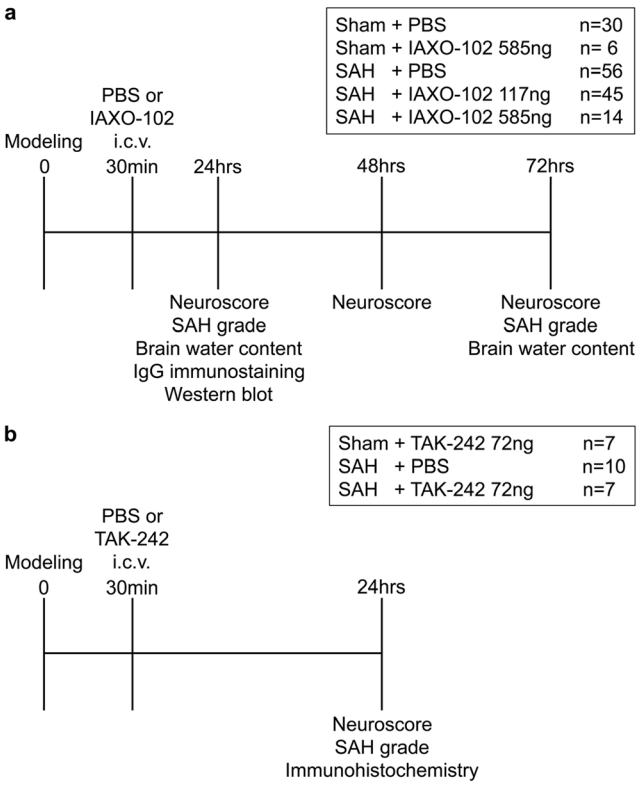

Fig. 1 Experimental designs. Experiment 1 (**a**) is designed to examine the effects of two dosages of a selective Toll-like receptor 4 (TLR4) antagonist IAXO-102 on blood-brain barrier (BBB) disruption after subarachnoid hemorrhage (SAH). Experiment 2 (**b**) is designed to confirm the effect of TLR4 blockage on post-SAH BBB disruption using another selective TLR4 antagonist TAK-242. *IgG* immunoglobulin G, *i.c.v.* intracerebroventricular infusion, *PBS* phosphate-buffered saline

Intracerebroventricular Infusion

An intracerebroventricular infusion was performed as previously described [18]. Briefly, the needle of a 2-μL Hamilton syringe (Hamilton Company, Reno, Nev) was inserted through a burr hole into the left lateral ventricle using the following coordinates relative to the bregma: 0.2 mm posterior, 1.0 mm lateral, and 2.2 mm below the horizontal plane of the bregma. Sterile vehicle (2 μL PBS) or TLR4 antagonists (117 or 585 ng IAXO-102 in 2 μL vehicle and 72 ng TAK-242 in 2 μL vehicle) were infused at a rate of 0.5 μL/min irrespective of the mouse's body weight at 30 min post-operation. The needle was removed at 10 min after an infusion, and the wound was quickly sutured.

The dosage of IAXO-102 was determined on the basis of our previous report [19]. In the study, 117 ng IAXO-102 was injected intracerebroventricularly to mice and significantly inhibited cerebral vasospasm after SAH. Assuming that a higher dosage of IAXO-102 should be needed to be effective for the brain, the dosage (117 ng) and a five-time higher dosage (585 ng) of IAXO-102 were tentatively selected in this study. The dosage of TAK-242 was determined on the basis of

a previous in vitro study [20]. In the study, 1 μmol/L TAK-242 completely inhibited the production of pro-inflammatory substances such as IL-6. The dosage of TAK-242 was calculated for working concentrations of 1 μmol/L and to achieve an equivalent cerebrospinal fluid (CSF) concentration in mice, whose total CSF volume is presumed to be 40 μL [21], and determined to be 72 ng. That is, 117 and 585 ng of IAXO-102 or 72 ng of TAK-242 were dissolved in 0.02 μL of 50% dimethyl sulfoxide and ethanol (volume ratio = 1:1), diluted 100 times in 1.98 μL of PBS (total solution volume = 2 μL, and final concentrations of dimethyl sulfoxide and ethanol = 0.5%, respectively) and injected.

Neurological Score

Neurological impairments were blindly evaluated using the modified Garcia's neurological score system as previously described [18]. The evaluation consisted of six categories (spontaneous activity; spontaneous movement of four limbs; forepaw outstretching; climbing; body proprioception; and response to whisker stimulation) that could be scored 0 to 3 or 1 to 3. Neurological score was determined by adding the six scores, with 2 being the worst and 18 the best. The median score of three consecutive trials in a 5-min interval was calculated.

SAH Severity

The severity of SAH was blindly assessed at each sacrifice as previously described [22]. The basal cistern was divided into six segments, and each segment was allotted a grade from 0 to 3 depending on the amount of SAH. The mice received a total score ranging from 0 to 18 by adding the scores from all six segments. In the analysis, mild SAH (SAH grade ≤ 7) mice were excluded because mild SAH did not cause neurological impairments [22].

Brain Water Content

Brain edema was determined using the wet/dry method as previously described [18]. After sacrificing mice under deep anesthesia, the brain was quickly removed, separated into four segments (left and right cerebral hemispheres, cerebellum, and brain stem), and weighted immediately as wet weight. The brain specimens were dried in an oven at 105 °C for 72 h and weighed again as dry weight. The water content of each specimen was calculated according to the following formula: [(wet weight − dry weight)/wet weight] × 100%.

Immunohistochemical Staining of Immunoglobulin G

Immunohistochemical staining of immunoglobulin G (IgG) was performed to evaluate BBB permeability as previously reported [18]. Under deep anesthesia, mice were sacrificed by transcardial perfusion with 30 mL PBS followed by 15 min of 10% neutral buffered formalin at 60–80 mmHg. After the brains were fixed in 10% neutral buffered formalin for 24 h at 4 °C and embedded in paraffin, 4-μL-thick coronal sections at 1.0 mm posterior to the bregma were cut. After being dewaxed, hydrated, and washed in distilled water, the sections were placed in 1 mmol/L ethylenediaminetetraacetic acid (EDTA; pH 8.0) heating in a water bath for 20 min to retrieve antigen. After being incubated in 3% hydrogen peroxide (H_2O_2) for 10 min to quench any endogenous peroxidase activity, the sections were blocked with 10% horse serum for 1 h at room temperature followed by overnight incubation at 4 °C with biotinylated anti-mouse polyclonal IgG (1:100; Vector Laboratories, Burlingame, CA) and then 1-hour incubation at room temperature with an avidin-biotin-horseradish peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA). Color reactions were developed in diaminobenzidine/H_2O_2 solution, and the sections were lightly counterstained with hematoxylin. To evaluate the amount of IgG extravasation, four continuous pictures of the left temporal cortex were photographed under a light microscope (× 20), and the relative quantity of extravasated IgG was calculated by Image Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD). The left (perforation side) temporal cortex was selected based on our previous study [18].

Immunohistochemical Staining of Activated JNK

Coronal brain sections were prepared in the same way as IgG staining. The staining procedure was performed using a commercially available kit (Mouse on Mouse Elite Peroxidase kit; Vector Laboratories, Burlingame, CA). After being dewaxed, hydrated, and washed in distilled water, the sections were placed in 1 mmol/L EDTA (pH 8.0) heating in a water bath for 20 min to retrieve antigen. After being incubated in 3% H_2O_2 for 10 min to quench any endogenous peroxidase activity, the sections were blocked with 5% horse serum followed by incubation overnight at 4 °C with mouse monoclonal anti-phosphorylated JNK antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA). Next, the sections were incubated with biotinylated anti-mouse IgG (1:250) for 10 min followed by incubated with an avidin-biotin complex for 5 min. Color reactions were developed in diaminobenzidine/H_2O_2 solution, and the sections were lightly counterstained with hematoxylin. The relative quantity of phosphorylated JNK was calculated by Image Pro Plus 6.0 software in the left temporal cortex as described in the above subsection for IgG staining.

Western Blotting

Western blotting was performed as previously described [19]. The left cerebral cortex was separated and used. Equal

amounts of protein samples (20 μg) were loaded on SDS-PAGE gels, electrophoresed, and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% bovine serum albumin for 4 h at room temperature followed by incubation overnight at 4 °C with rabbit polyclonal anti-TLR4 antibody (1:500; Abcam, Cambridge, UK), mouse monoclonal anti-phosphorylated p38 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit monoclonal anti-phosphorylated ERK1/2 antibody (1:2000; Cell Signaling Technology, Danvers, MA), mouse monoclonal anti-phosphorylated JNK antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit monoclonal anti-IL-6 antibody (1:1000; Cell Signaling Technology, Danvers, MA), anti-TNC antibody (1:8000; Immuno-Biological Laboratories, Takasaki, Japan), anti-periostin antibody (1:8000; Abcam, Cambridge, UK), rabbit polyclonal anti-MMP-9 antibody (1:1000; Merck Millipore, Temecula, CA), and rabbit polyclonal anti-zona occludens (ZO)-1 antibody (1:200; Invitrogen, Carlsbad, CA). Immunoreactive bands were detected with a chemiluminescence reagent kit (ECL Prime; Amersham Bioscience, Arlington Heights, IL) and quantified by densitometry with Image J software (NIH, Bethesda, MD). β-tubulin (1:1000; Cell Signaling Technology, Danvers, MA) was used as an internal control for every procedure. Changes in the protein expression were expressed as a ratio of the protein values/values of β-tubulin in sham-operated mice treated with PBS at 24 h.

Statistical Analysis

Neurological score and SAH grade were expressed as median ± 25th–75th percentiles and were compared with Mann-Whitney *U* tests or Kruskal-Wallis tests followed by Steel-Dwass multiple comparisons. Brain water content, IgG immunostaining, and Western blotting results were expressed as mean ± standard deviation and were compared with one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc tests. Mortality was compared using Fisher's exact tests, and survival rates were compared using log-rank tests with Bonferroni correction. Statistical analyses were performed using SPSS version 24.0 (IBM, Tokyo, Japan). A value of $p < 0.05$ was considered significant.

Results

TLR4 Antagonist Improves Survival Rates, Neurological Scores, and Brain Edema

Comparisons of physiological parameters revealed no significant differences among the groups (data not shown). No sham-operated mice died during the observation period. The mortality of SAH mice after randomization into each treatment group was not significantly different among the groups at 24 h: 9 of 56 mice (16.1%) in the SAH + PBS group, 5 of 45 (11.1%) in the SAH + IAXO-102 117 ng group, and 1 of 14 (7.1%) in the SAH + IAXO-102 585 ng group. At 24 h, SAH grades were mild (≤ 7) in 12 mice in the SAH + PBS group, 10 mice in the SAH + IAXO-102 117 ng group, and 5 mice in the SAH + IAXO-102 585 ng group, which were not used for the analyses (Online Resource, Table S1). SAH grading scores of the remaining animals were similar among the groups at 24 h post-SAH (Fig. 2a). IAXO-102 administration neither affected neurological scores nor brain edema in sham-operated mice (Fig. 2b, c). However, post-SAH administration of both dosages of IAXO-102 significantly prevented post-SAH aggravation of neurological scores (Fig. 2b) and brain water content in the left cerebral hemisphere at 24 h post-SAH (Fig. 2c) equally. Neither SAH nor IAXO-102 administration affected brain water content in the right cerebral hemisphere, cerebellum, and brain stem (Online Resource, Fig. S1). Therefore, the following experiments were performed using the sham + PBS, SAH + PBS, and SAH + IAXO-102 117 ng groups.

During the period from > 24 to 72 h post-SAH, six mice died only in the SAH + PBS group (Online Resource, Table S1). Kaplan-Meier survival curves demonstrated that survival rates were not different between the sham + PBS and SAH + IAXO-102 117 ng groups, but those of the SAH + PBS group were significantly worse compared with the sham + PBS group ($p < 0.001$; Fig. 3). At 72 h, as one mouse in the SAH + PBS and the SAH + IAXO-102 117 ng groups, respectively, had mild SAH grades ≤ 7, they were not used for the analyses. SAH grades of the remaining animals were similar between the groups (Online Resource, Fig. S2a). IAXO-102 also inhibited post-SAH aggravation of neurological scores and brain edema in the left cerebral hemisphere, but the difference did not reach significance between the SAH + PBS and the SAH + IAXO-102 117 ng groups at 72 h (Online Resource, Fig. S2 b and c). Neither SAH nor IAXO-102 administration affected brain water content in the right cerebral hemisphere, cerebellum, and brain stem (Online Resource, Fig. S2 d–f).

TLR4 Antagonist Prevents Post-SAH BBB Disruption

SAH resulted in a significant increase in IgG extravasation at 24 h post-SAH, which was significantly suppressed by IAXO-102 administration ($p < 0.05$; Fig. 4). Western blotting showed that IAXO-102 prevented post-SAH TLR4 induction and that the expression levels of TLR4 were not different between the sham + PBS and the SAH + IAXO-102 117 ng groups (Online Resource, Fig. S3). Western blot analyses also demonstrated that the protective effects by IAXO-102 were associated with a significant inhibition of post-SAH activation of a proteolytic enzyme MMP-9 and the consequent preservation of tight junction protein ZO-1 (Fig. 5).

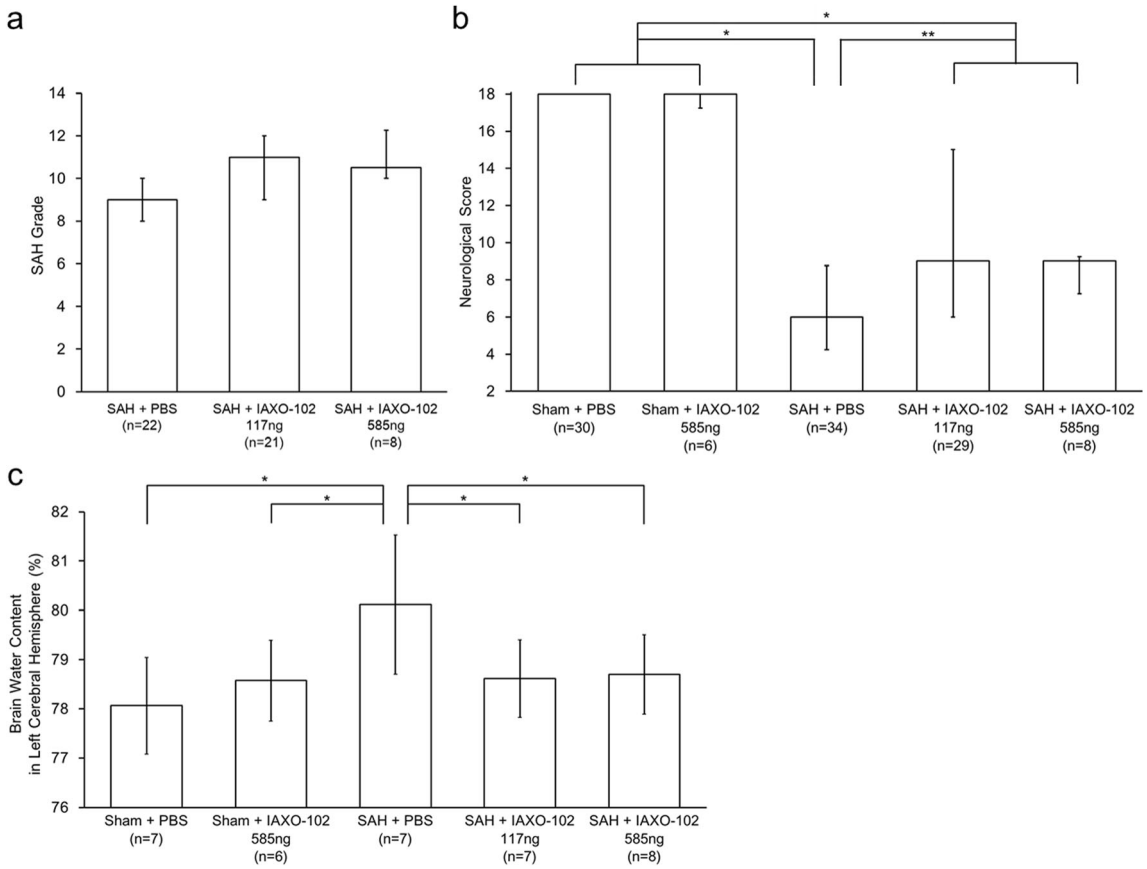

Fig. 2 Effects of a selective Toll-like receptor 4 antagonist IAXO-102 on the severity of subarachnoid hemorrhage (SAH) (**a**), neurological scores (**b**), and brain water content in the left cerebral hemisphere (**c**) at 24 h after SAH. Data are expressed as median ± 25th–75th percentiles (**a**, **b**) and mean ± standard deviation (**c**); $*p < 0.001$, $**p < 0.05$, Kruskal-Wallis tests (**a**, **b**), and ANOVA (**c**). *PBS* phosphate-buffered saline

JNK and Periostin May Make a Greater Contribution to TLR4-Mediated BBB Disruption

As intracellular mediators of TLR4 signaling, activation of p38, ERK1/2, and JNK was evaluated by Western blotting. SAH significantly activated p38 and JNK but not ERK1/2 (Online Resource, Fig. S4). IAXO-102 suppressed both p38 and JNK activation after SAH, but only p46 JNK was significantly inactivated in the SAH + IAXO-102 117 ng group ($p < 0.05$ versus SAH + PBS). Expression levels of downstream inflammatory mediators of TLR4 signaling pathways, IL-6, periostin, and TNC, were also significantly increased by SAH and suppressed by IAXO-102; the differences were significant only in periostin ($p < 0.05$, SAH + PBS versus SAH + IAXO-102 117 ng; Online Resource, Fig. S5).

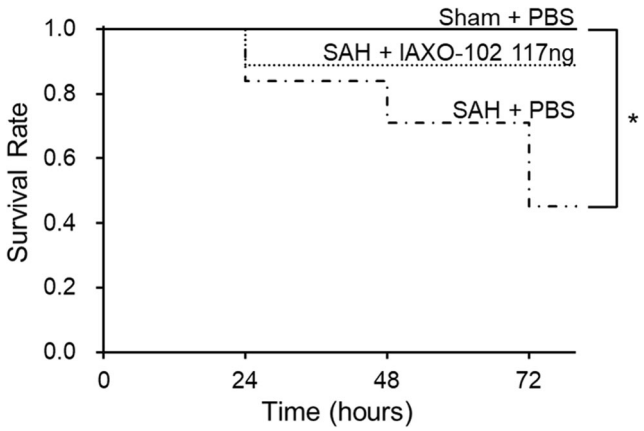

Fig. 3 Kaplan-Meier survival curves of sham-operated mice treated with phosphate-buffered saline (PBS) and subarachnoid hemorrhage (SAH) mice treated with a selective Toll-like receptor 4 antagonist IAXO-102 or PBS. $*p < 0.001$, log-rank tests

Another TLR4 Antagonist TAK-242 also Prevents Post-SAH JNK Activation and BBB Disruption

Comparisons of physiological parameters revealed no significant differences among the groups (data not shown). After randomization into each treatment group, no mice died during the 24-h observation period. As the severity of SAH was mild (SAH grades ≤ 7) in four mice in the SAH + PBS group and two mice in the SAH + TAK-242 72 ng group, the remaining animals were used for the analyses (Online Resource, Table S1). The median SAH grading score was similar between the groups (Fig. 6a). TAK-242 treatment had no effects on neurological scores in sham-operated mice but significantly

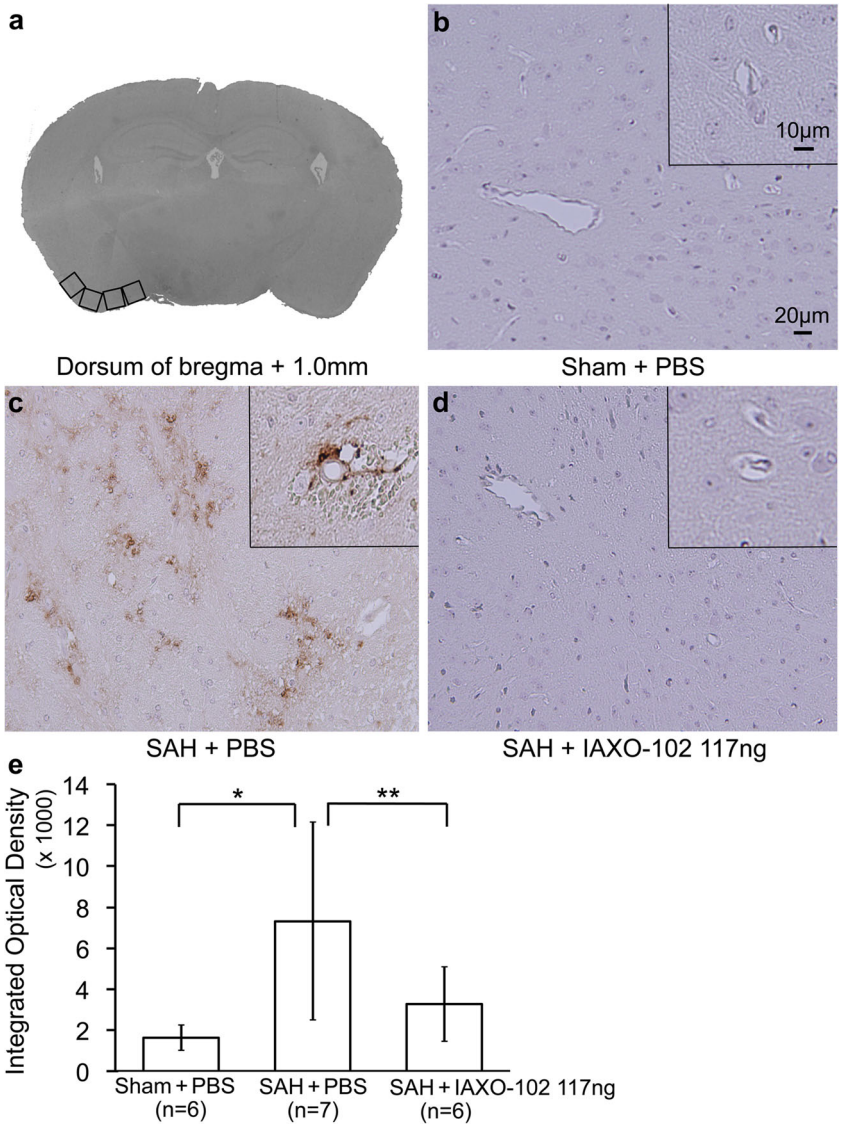

Fig. 4 Effects of a selective Toll-like receptor 4 antagonist IAXO-102 on blood-brain barrier permeability in the left temporal cortex at 24 h after subarachnoid hemorrhage (SAH). Representative brain slice showing four continuous areas in the left temporal cortex at 1.0 mm posterior to the bregma (**a**), representative immunohistochemical staining of immunoglobulin G (IgG) in sham-operated mice treated with phosphate-buffered saline (PBS) (**b**), SAH mice treated with PBS (**c**), and SAH mice treated with IAXO-102 (**d**) and the sum of integrated optical density of IgG (**e**). Data are expressed as mean ± standard deviation; $*p < 0.001$, $**p < 0.05$, ANOVA

prevented post-SAH neurological impairments ($p < 0.05$; Fig. 6b). IgG extravasation was also significantly inhibited in the SAH + TAK-242 72 ng group compared with the SAH + PBS group ($p < 0.05$; Fig. 7).

Immunohistochemical staining showed that the immunoreactivities of phosphorylated JNK were increased in the capillary endothelial cells in the left temporal cortex in the SAH + PBS group but were attenuated in the SAH + TAK-242 72 ng group (Fig. 8).

Discussion

The novel findings in the present study are as follows: (1) a selective TLR4 antagonist IAXO-102 improved survival rates and neurological scores in endovascular perforation SAH mice; (2) two kinds of selective TLR4 antagonists suppressed post-SAH BBB disruption as measured by IgG extravasation; and (3) selective blockage of TLR4 also prevented JNK activation, MMP-9 activation, and periostin induction after SAH. This is the first study demonstrating the direct linkage between TLR4 and BBB disruption after SAH, as well as the involvement of TLR4/MAPK signaling in post-SAH BBB disruption.

TLR4 is expressed in various cells including brain capillary endothelial cells in the central nervous system and can be activated by many endogenous ligands having damage-associated molecular patterns including heme, fibrinogen, inflammatory mediators, and intracellular components of ruptured cells, all of which are abundantly produced after SAH [5]. TLR4 activation by ligands needs its extracellular binding partner myeloid differentiation factor-2 (MD-2) and a cluster of differentiation 14 (CD14) to activate NF-κB and MAPK downstream pathways, both of which lead to the production of pro-inflammatory mediators and may cause post-SAH EBI [5, 9]. Some studies reported that nonspecific TLR4 inhibitors alleviated post-SAH BBB disruption [5]. For example, tamoxifen, a selective

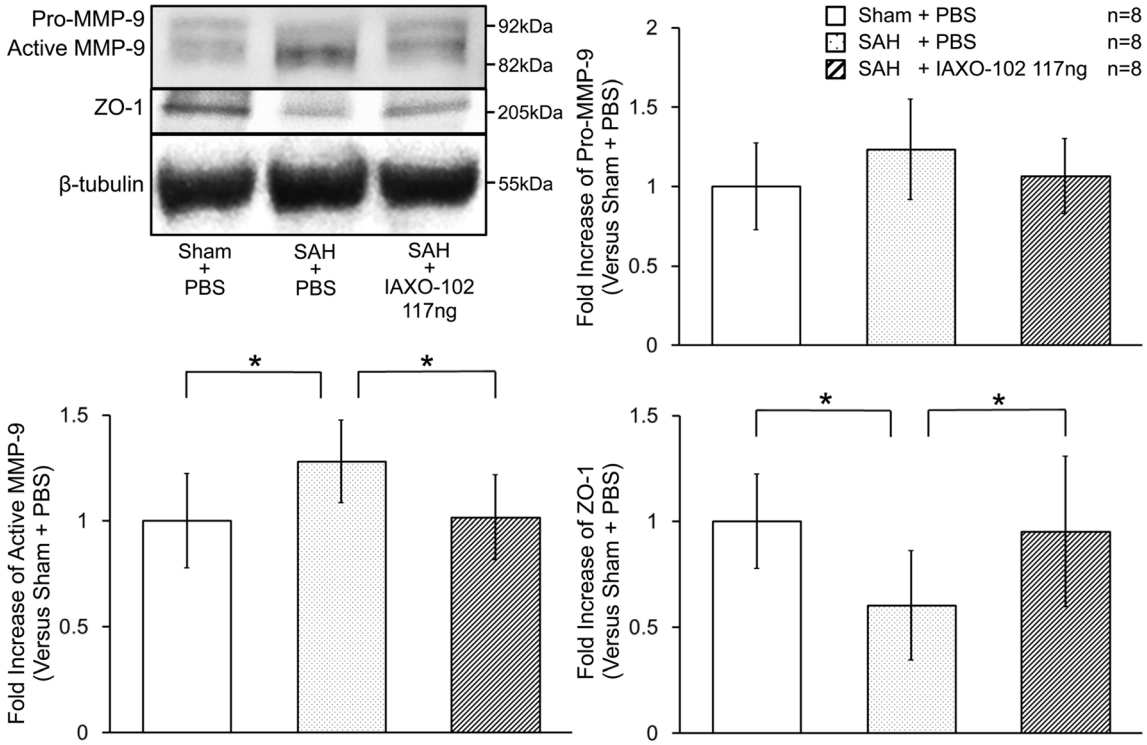

Fig. 5 Representative Western blots and effects of a selective Toll-like receptor 4 antagonist IAXO-102 on the expression of matrix metalloproteinase (MMP)-9 and zona occludens (ZO)-1 in the left cerebral cortex at 24 h after subarachnoid hemorrhage (SAH). Representative Western blots come from the same samples but two membranes. Expression levels of each protein are expressed as a ratio of β-tubulin levels for normalization and as mean ± standard deviation; $*p < 0.05$, ANOVA. *PBS* phosphate-buffered saline

estrogen receptor modulator, and resveratrol, a kind of antioxidant, suppressed post-SAH BBB disruption and neuroinflammation, associated with inactivation of TLR4/NF-κB signaling pathways in rats [16, 17]. As far as we know, however, no studies have investigated the effects of selective TLR4 antagonists against post-SAH EBI including BBB disruption and possible involvement of TLR4/MAPK signaling pathways in post-SAH EBI including BBB disruption. In this study, two kinds of selective TLR4 antagonists, IAXO-102 and TAK-242, were used and first demonstrated that selective blockage of TLR4 prevented post-SAH BBB disruption associated with inactivation of JNK, a major isoform of MAPK. IAXO-102 interferes CD14 selectively with higher affinity than the TLR4 co-receptors MD-2 by its lipid A structure, while TAK-242 is an inhibitor with a chemical structure totally different from lipid A, and selectively inhibits TLR4 signaling by covalently binding to a specific amino acid cysteine 747 in the intracellular domain of TLR4 [23, 24].

BBB is mainly composed of a chemical and structural barrier formed by endothelial tight junction-associated proteins, such as occludin and ZO-1 [10, 11]. Degradation of these proteins opens tight junction and increases BBB permeability [11, 15]. Among pro-inflammatory mediators that can be induced by TLR4 signaling, MMP-9 has been repeatedly

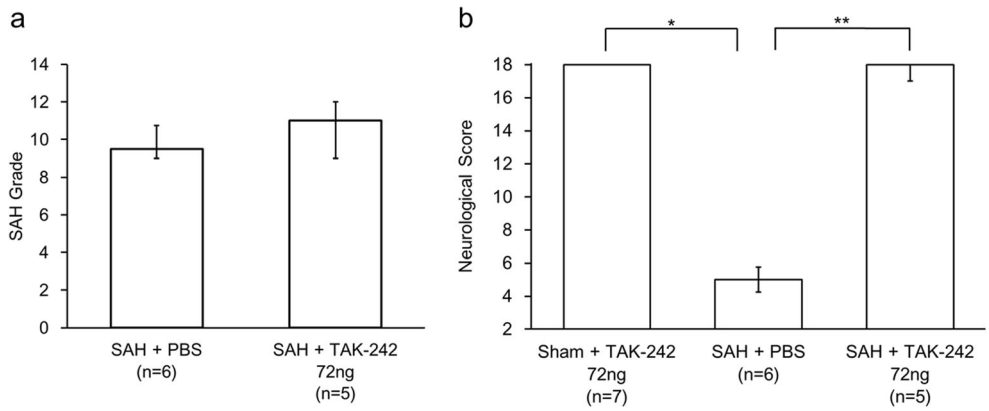

Fig. 6 Effects of a selective Toll-like receptor 4 antagonist TAK-242 on the severity of subarachnoid hemorrhage (SAH) (**a**) and neurological scores (**b**) at 24 h after SAH. Data are expressed as median ± 25th–75th percentiles; $*p < 0.001$, $**p < 0.05$, Mann-Whitney *U* test (**a**) and Kruskal-Wallis test (**b**). *PBS* phosphate-buffered saline

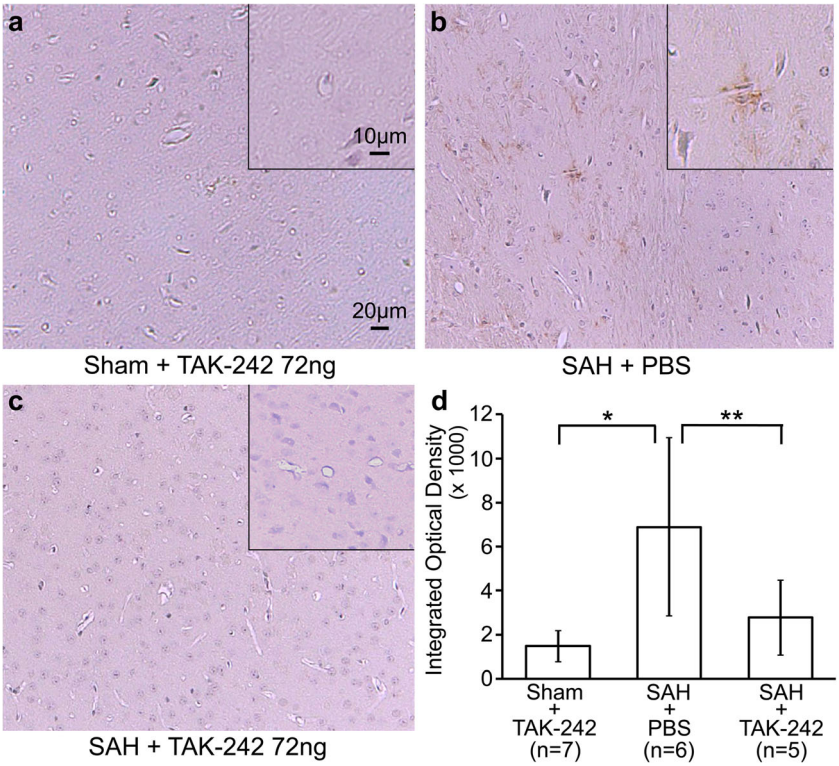

Fig. 7 Effects of a selective Toll-like receptor 4 antagonist TAK-242 on blood-brain barrier permeability in the left temporal cortex at the dorsum of bregma + 1.0 mm at 24 h after subarachnoid hemorrhage (SAH). Representative immunohistochemical staining of immunoglobulin G (IgG) in sham-operated mice treated with TAK-242 (**a**), SAH mice treated with phosphate-buffered saline (PBS) (**b**), and SAH mice treated with TAK-242 (**c**) and the sum of integrated optical density of IgG (**d**). Data are expressed as mean ± standard deviation; *$p < 0.001$, **$p < 0.05$, ANOVA

reported to cause post-SAH BBB disruption by degrading the extracellular matrix proteins of the cerebral microvessel basal lamina and interendothelial tight junction proteins such as ZO-1 [13, 25–28]. MMP-9 knockout was demonstrated to inhibit post-SAH brain edema formation and neurological impairments in mice [28]. In addition, other TLR4 signaling

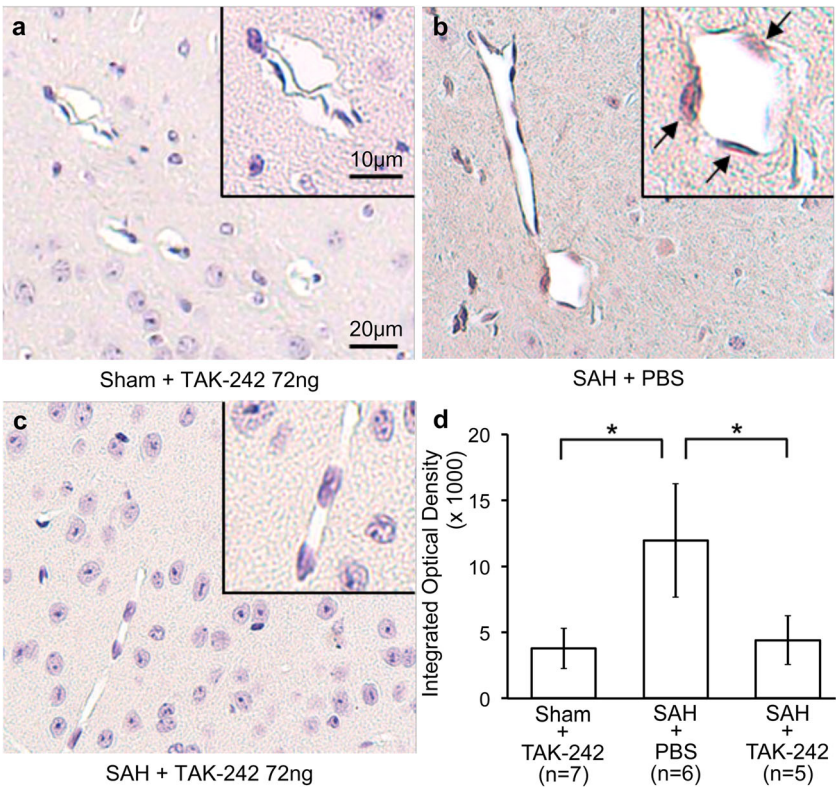

Fig. 8 Effects of a selective Toll-like receptor 4 antagonist TAK-242 treatment on the expression of phosphorylated c-Jun N-terminal kinase (p-JNK) in the left temporal cortex at 1.0 mm posterior to the bregma at 24 h post-subarachnoid hemorrhage (SAH). Representative immunohistochemical staining of p-JNK in sham-operated mice treated with TAK-242 (**a**), SAH mice treated with phosphate-buffered saline (PBS) (**b**), and SAH mice treated with TAK-242 (**c**) and the sum of integrated voptical density of p-JNK (**d**). The arrows indicate immunoreactive endothelial cells. Data are expressed as mean ± standard deviation; *$p < 0.001$, ANOVA

products, periostin, TNC, and IL-6 also can trigger a cascade that leads to BBB disruption via MMP-9 upregulation and activation [5, 11, 13, 29]. Periostin and TNC are matricellular proteins and directly interact with each other, causing post-SAH BBB disruption [5, 11]. MAPKs are both downstream and upstream of periostin, TNC, and IL-6, that is, activated MAPKs induce periostin, TNC, and IL-6, which in turn activate MAPKs, forming a positive feedback mechanism to cause and aggravate post-SAH brain injuries via the mechanisms including MMP-9 activation [5, 10–15, 30]. Our recent study demonstrated that post-SAH induction of TNC caused BBB disruption by activating three major isoforms of MAPKs (JNK, ERK1/2, and p38) and then MMP-9 in the brain capillary endothelial cells in endovascular perforation SAH mice [13]. Activation of JNK and p38 was also implicated in TNC-induced TLR4 upregulation in a rat cerebral artery, which was abolished by a selective TLR4 antagonist LPS-RS, selective inhibitors of JNK, and p38, respectively [31]; these findings can explain the findings in this study that a selective TLR4 antagonist IAXO-102 suppressed post-SAH upregulation of TLR4 associated with JNK inactivation. Thus, it may be reasonable to consider that post-SAH TLR4-meadiated BBB disruption involves activation of MAPKs and upregulation of pro-inflammatory mediators such as MMP-9, IL-6, periostin, and TNC, as well as TLR4 itself. However, another study reported that post-SAH periostin induction interacted with TNC; activated ERK1/2, p38, and MMP-9, but not JNK; and caused BBB disruption in endovascular perforation SAH mice [11]. In this study, using the same animal models, JNK activation and periostin induction were shown to make greater contributions to TLR4-mediated MMP-9-induced BBB disruption, while the role of activation of p38 or ERK1/2 and induction of IL-6 or TNC seemed to be limited. Differences in expressions of MAPKs or other pro-inflammatory mediators in post-SAH brain may be explained by limited injury with an irregular pattern in the cerebral cortex, which also might cause failed detection of significant differences in expression levels of TLR4, phosphorylated p38, IL-6, and TNC between SAH mice treated with vehicle and TLR4 antagonists in this study.

This study is somewhat limited. First, TLR4 antagonists were administered intracerebroventricularly. To be more translational, other treatment routs such as intravenous injections should be tested. Second, only immediate treatment with TLR4 antagonists at 30 min after SAH was tested in this study, showing that the 72-h effects were limited. This may mean that the treatment regimen in this study should be improved, and the effects of multiple treatments at different dosages or time courses as well as long-term functional outcomes should be examined in future preclinical evaluations [32]. Third, this study used two kinds of TLR4 antagonists. To examine which one is more effective against post-SAH BBB disruption before clinical trials, many meticulous studies are needed [32]. Lastly, this study demonstrated that two kinds of selective TLR4 antagonists blocked post-SAH BBB disruption as well as post-SAH activation of JNK a major isoform of MAPK using two methods, Western blotting, and immunohistochemistry. However, the mechanisms for TLR4 activation to induce post-SAH BBB disruption were not completely unveiled including the role of MAPK, NF-κB, and other downstream pro-inflammatory mediators that were not examined in this study. Further clarification of the mechanisms between signal activation of TLR4 and post-SAH BBB disruption is expected. However, the present study provided new evidence suggesting the importance of TLR4 signaling as a potential molecular target for therapy against post-SAH EBI as to BBB disruption.

In conclusion, this study demonstrated for the first time that selective blockage of TLR4 prevented post-SAH BBB disruption possibly via the inhibition of JNK activation, MMP-9 activation, and periostin induction in mice.

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Compliance with Ethical Standards

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

Research Involving Animals All procedures were approved by the Animal Ethics Review Committee of Mie University and were carried out according to the institution's Guidelines for Animal Experiments.

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