Inhibition of Leukotriene B4 Action Mitigates Intracerebral Hemorrhage-Associated Pathological Events in Mice

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**Abbreviations:** 5-LOX, 5-lipoxygenase; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; FLAP, 5-lipoxygenase-activating protein; ICH, intracerebral hemorrhage; LTA₄H, leukotriene A₄ hydrolase; LTB₄, leukotriene B₄; MPO, myeloperoxidase; MRI, magnetic resonance imaging; NF-H, neurofilament-H; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; TNF-α, tumor necrosis factor α; WT, wild-type.

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Abstract

Infiltration of neutrophils has been suggested to play an important role in the pathogenesis of intracerebral hemorrhage (ICH) for which effective therapeutic interventions remain unavailable. In the present study we focused on leukotriene B4 (LTB4) as a potent chemotactic factor for neutrophils, to address its contribution to the pathological events associated with ICH. ICH with hematoma expansion into the internal capsule that resulted in severe sensorimotor dysfunction was induced by injection of collagenase in mouse striatum. We found that LTB4 as well as mRNAs of 5-lipoxygenase and 5-lipoxygenase-activating protein was increased in the brain after ICH. Daily treatment with a 5-lipoxygenase inhibitor zileuton (3 or 10 mg/kg, i.v.) prevented ICH-induced increase in LTB4, attenuated neutrophil infiltration into the hematoma, and ameliorated sensorimotor dysfunction. In addition, mice deficient for LTB4 receptor BLT1 exhibited lower number of infiltrating neutrophils in the hematoma and lower levels of sensorimotor dysfunction after ICH than wild-type mice. Similarly, daily treatment of mice with a BLT antagonist ONO-4057 (30 or 100 mg/kg, p.o.) from 3 h after induction of ICH inhibited neutrophil infiltration and ameliorated sensorimotor dysfunction. ONO-4057 also attenuated inflammatory responses of microglia/macrophages in the perihematoma region and axon injury in the internal capsule. These results identify LTB4 as a critical factor that plays a major role in the pathogenic events in ICH, and propose BLT1 as a promising target for ICH therapy.
Introduction

Intracerebral hemorrhage (ICH) is characterized by rupture of blood vessels and formation of hematoma within the brain parenchyma, which leads to high mortality, coma and long-lasting hemiplegia (Qureshi et al., 2009). Because effective pharmacotherapies are currently unavailable (Katsuki, 2010), identification of potential drug targets may provide novel opportunities for alleviating severe prognosis of this neurological disorder.

As in the case with other neurological disorders associated with neurodegeneration, ICH is accompanied by various inflammatory events, within and around hematoma. These events include infiltration of neutrophils, monocytes and T cells, and proinflammatory activation of resident microglia and infiltrating monocytes/macrophages (Hijioka et al., 2011; Zhou et al., 2014). Of these, neutrophils may play a key role in the brain tissue damage leading to neurological dysfunction (Mracsko et al., 2014). This point of view is supported by a study that addressed the effect of neutrophil depletion on the pathological consequences of ICH. That is, depletion of circulating neutrophils by pretreatment with anti-polymorphonuclear antigen diminished pathological changes and ameliorated neurological dysfunction in a rat model of ICH (Moxon-Emre and Schlichter, 2011). Therefore, prevention of neutrophil infiltration may present a promising strategy for ICH therapy. However, detailed cellular mechanisms involved in neutrophil infiltration under the conditions of ICH have not been revealed so far.

Leukotriene B4 (LTB4) is an arachidonic acid metabolite that is generated through several biosynthetic steps initiated by 5-lipoxygenase (5-LOX). This lipid mediator is well known to act as a potent chemoattractant for neutrophils and other leukocytes (Yokomizo et al., 1997, 2001). Receptors for LTB4 are designated as BLT1 and BLT2, and the former is the high affinity receptor for LTB4 that is expressed in inflammatory and immune cells including leukocytes and mediates chemotaxis (Yokomizo et al., 1997). Accumulating lines of evidence suggest that LTB4 production and BLT1 activation play a critical role in neutrophil infiltration and inflammatory pathogenesis in several types of peripheral and central nervous system disorders (Kihara et al., 2010; Saiwai et al., 2010; Asahara et al., 2015). In the case of
ischemia-reperfusion of middle cerebral artery, 5-LOX inhibition suppresses production of LTB₄ and attenuates brain injury, although the role of BLT₁ has not been examined (Tu et al., 2010).

To date, the role of LTB₄ and BLT₁ in the pathological events in ICH has not been addressed. Notably, however, microarray analysis of the brain tissues from human ICH patients has detected increased expression of mRNAs encoding 5-LOX (ALOX5 mRNA) and 5-LOX-activating protein FLAP (ALOX5AP mRNA) (Carmichael et al., 2008). Together with the fact that ICH is accompanied by a robust increase in infiltrating neutrophils (Zhou et al., 2014), this line of evidence suggests that LTB₄ plays an important role in the pathogenesis of ICH. In the present study we addressed the pathogenic role of LTB₄, by using mouse model of ICH with injury of the internal capsule that resulted in severe sensorimotor deficits (Matsushita et al., 2013).

Materials and Methods

Animals. All procedures were approved by the Kumamoto University ethics committee on animal experiments, and animals were treated in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Male C57BL/6J mice, BLT₁-deficient (BLT₁-KO) mice (Terawaki et al., 2005) and wild-type (WT) littermates of C57BL/6 background at 8 to 10 weeks of age weighing 22 to 30 g were used. Animals were maintained at constant ambient temperature (22 ± 1 °C) under a 12-h light/dark cycle (lights on between 8:00 AM and 8:00 PM) with food and water available ad libitum.

Induction of ICH. ICH model was prepared as described previously (Matsushita et al., 2013). After anesthesia with pentobarbital (50 mg/kg, i.p.), 0.025 U of collagenase type VII (Sigma-Aldrich, St. Louis, MO, USA) in 0.5 μl saline was injected at 0.2 μl/min (stereotaxic coordinates: 2.3 mm lateral to the midline, 0.2 mm posterior to the bregma, and 3.5 mm deep below the skull). Because the site of collagenase injection was located adjacent to the internal capsule, hemorrhage expanded into the internal capsule, damaged the corticospinal tract and produced severe sensorimotor dysfunction (Matsushita et al., 2013). We did not observe any pain-related behaviors in mice after surgery for ICH induction, and no analgesics
were used in the present study.

**Drug Treatment.** Zileuton (1-[1-(benzo[b]thiophen-2-yl)ethyl]-1-hydroxyurea; LKT Laboratories, St. Paul, MN, USA) was dissolved in 25% dimethyl sulfoxide-containing saline at 0.6 or 2.0 mg/ml and administered intravenously at daily doses of 3 or 10 mg/kg, with the first injection given at 15 min after induction of ICH. Control animals (drug at 0 mg/kg) received administration of vehicle (25% dimethyl sulfoxide-containing saline) at the same schedule as that of drug treatment groups. ONO-4057 (5-[2-(2-carboxyethyl)-3-[[E]-6-(4-methoxyphenyl)-5-hexenyl]oxy]phenoxy]pentanoic acid; provided by Ono Pharmaceutical Co., Ltd., Osaka, Japan), an LTB₄ receptor antagonist (Kishikawa et al., 1992), was dissolved in 0.5% carboxymethylcellulose at 1.5 or 5.0 mg/ml and orally administered at daily doses of 30 or 100 mg/kg, with the first dose given at 3 h after ICH induction. Control animals (drug at 0 mg/kg) received administration of vehicle (0.5% carboxymethylcellulose solution) at the same schedule as that of drug treatment groups.

**Enzyme-Linked Immunosorbent Assay (ELISA).** Mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and transcardially perfused with 30 ml cold phosphate-buffered saline. The brain was removed from the skull, the olfactory bulb was excised, and a coronal section of 4 mm thickness was obtained from the anterior end of the brain tissue. The hemisphere containing the entire hematoma region was used for the analysis. Quantification of LTB₄ content in the brain tissue was performed with a competitive ELISA kit (EA35 Leukotriene B₄ EIA kit; Oxford Biomedical Research, Oxford, MI, USA). According to the material sheet, cross-reactivity of the assay to LTB₄ metabolites such as 20-hydroxy-LTB₄ and 20-carboxy-LTB₄ is 0.5% and < 0.10%, respectively. Extraction of LTB₄ was performed according to the standard protocol given in the material sheet. Briefly, samples of homogenized brain tissues were extracted using C18 octadecyl minicolumns (C18 Sep-Pak® Light column; Waters Corporation, Milford, MA, USA), eluted with methyl formate, and evaporated with N₂ gas. LTB₄-containing samples were resuspended in the assay buffer. ELISA was carried out in triplicate for each sample.

**Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).** Brain tissues were
obtained in the same manner as that described above. qRT-PCR was performed with SYBR® Premix Ex Taq™ (TaKaRa Bio, Kusatsu, Japan) on the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Tokyo, Japan). The thermal cycling program consisted of 95 °C for 3 min for polymerase activation, and then 40 cycles of denaturation (95 °C for 15 s) and annealing and extension (60 °C for 1 min). Reactions were quantified by selecting the amplification cycle when the PCR product of interest was first detected [the threshold cycle (Ct)]. Data were analyzed by the comparative Ct method. Primer sequences are listed in Table 1.

**Immunohistochemical examinations.** Immunohistochemical analyses were performed on frozen coronal sections with thickness of 30 μm, prepared as described previously (Hijioka et al., 2016). In the experiments for quantification of neutrophil invasion, microglial activation and axonal injury, rabbit-anti myeloperoxidase (MPO) (1:500, Dako, Glostrup, Denmark), rabbit anti-Iba1 (1:1,000, Wako Chemicals, Osaka, Japan) and mouse anti-neurofilament H (NF-H) (1:500, Cell Signaling Technology, Danvers, MA, USA) were used as the primary antibodies, respectively. Alexa Fluor 594 conjugated donkey anti-rabbit IgG (H+L) (1:1,000, Invitrogen™, Life Technologies Japan, Tokyo, Japan) and Alexa Fluor 488-conjugated donkey anti-mouse IgG (H+L) (1:1,000, Invitrogen™) were used as the secondary antibodies. Fluorescence images were obtained with the Fluoview FV300 system (Olympus, Tokyo, Japan) or BIOREVO BZ-9000 (Keyence, Osaka, Japan). MPO-positive cells in the central region of the hematoma were counted from the images of 367 × 276 μm². Threshold-based quantification of Iba1-positive area in the peripheral region of the hematoma was conducted with the NIH ImageJ software, in arbitrary fields from the fluorescence images of 725 × 546 μm². Comparative analysis of the morphological changes in axonal fibers was made with NIH ImageJ software as described in our previous study (Hijioka et al., 2016). Fifteen fibers in an image of 120 × 120 μm² were randomly selected for the measurement.

**Assessment of sensorimotor dysfunction.** Sensorimotor functions of mice were evaluated by the modified limb-placing test and the beam-walking test at 6, 24, 48 and 72 h after induction of ICH. These
tests were conducted by an experimenter blinded to the treatments. The modified limb-placing test consisted of two limb-placing tasks that assessed the sensorimotor integration of the forelimb and the hindlimb by testing the responses to tactile and proprioceptive stimuli. Details for scoring have been described in our previous study (Matsushita et al., 2011), and the maximal deficit score in individual mice was 7. In the beam-walking test, mice were trained once daily for 3 days before the surgery. Mice were placed on a beam (1.1 m in length, 1.5 cm in width, and 50 cm in height), and usage of the hind limb during beam crossing was analyzed as a fault rate. The fault rate was presented as an average from three trials.

Magnetic resonance imaging (MRI). Three days after ICH induction, mice were anesthetized with isoflurane and scanned with Biospec 7-Tesla 70/20 USR (Bruker Biospin KK, Yokohama, Japan) with mouse brain surface coil as described previously (Matsushita et al., 2013, 2014). T2-weighted images (turbo RARE pulse sequence, TR 3839.5 ms, TE 47.6 ms, FOV: 2.5 × 2.5 cm, matrix: 500 × 500, RARE factor 8, 25 slices, 0.5 mm thickness) were acquired. The hematoma volume was determined by integration of the lesioned area in each section over the section depth, with the use of OsiriX Lite software (Pixmeo, Geneva, Switzerland).

Statistics. All data are presented as means ± S.E.M. Statistical analyses were carried out with the GraphPad Prism 6 software (Graph Pad, San Diego, CA, USA). For two-group comparison, data were analyzed by Student’s *t*-test. If the distribution of data points was not suitable for *t*-test, non-parametric Mann-Whitney *U*-test was used. When the data sets included more than two groups, one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test was employed. Non-parametrical Kruskal-Wallis test followed by Dunn’s multiple comparisons test was used when the distribution of data points was not suitable for one-way ANOVA. Data on neurological function were analyzed by two-way ANOVA with repeated measures, followed by post-hoc comparisons with Bonferroni method. In all cases, two-tailed probability values less than 0.05 were considered significant.

Results
Expression of 5-LOX and 5-LOX-activating protein is increased in the brain after ICH. First we examined the changes in the expression levels of mRNAs encoding key molecules involved in LTB₄ production. ALOX5 mRNA encoding 5-LOX, the rate-limiting enzyme for LTB₄ production, was increased in the brain from 18 h after ICH induction (Fig. 1A). In addition, ALOX5AP mRNA encoding FLAP was increased from 24 h after ICH induction (Fig. 1B). On the other hand, the mRNA expression level of LTA₄ hydrolase (LTA₄H) that converts LTA₄ into LTB₄ did not show significant change after ICH (Fig. 1C). We also found that the expression of mRNA for LTB₄ ω-hydroxylase (CYP4F14), an enzyme metabolizing LTB₄, was significantly increased at 48 h after ICH or later (Fig. 1D).

5-LOX inhibitor diminishes neutrophil invasion and alleviates ICH-induced sensorimotor deficits. Next we examined the effect of zileuton, a 5-LOX inhibitor (Carter et al., 1991), on the pathological events in ICH. This drug has been shown to inhibit whole blood LTB₄ synthesis in the rat with an EC₅₀ of 2 mg/kg, p.o. (Carter et al., 1991), and was used in another study on renal ischemia-reperfusion injury in mice at 3 mg/kg, i.v. (Patel et al., 2004). Competitive ELISA revealed that LTB₄ content in the brain tissues was increased significantly at 24 h, but not 6 h, after induction of ICH as compared to the sham control (Fig. 2A and B). The increase in LTB₄ content was transient, and there was no significant difference between the sham control group (9.51 ± 2.13 ng/mg tissue, n = 5) and the ICH group (9.58 ± 2.24 ng/mg tissue, n = 5) at 72 h after induction of ICH. When zileuton (3 or 10 mg/kg) was administered intravenously once daily for 3 days starting from 15 min after induction of ICH, the drug abolished the increase of LTB₄ at 24 h after ICH. Under the same conditions, zileuton did not affect the expression level of ALOX5 mRNA assessed at 72 h after ICH induction, namely, even after repeated administration for three times (Fig. 2C).

Neutrophil invasion into the brain was assessed by MPO immunohistochemistry. No MPO-immunopositive signals were observed in the brain of sham-operated mice. In contrast, we observed a small number of infiltrating neutrophils at 6 h after ICH induction, and numerous neutrophils were present in the hematoma at 24 h after ICH. The number of neutrophils at 72 h after ICH was comparable to that of 24 h, which seemed to reach plateau level at this time point (Fig. 2D). Treatment with zileuton (3 or 10
mg/kg) partially but significantly attenuated the increase in the number of neutrophils in the hematoma at 72 h (Fig. 2E, F).

To address whether 5-LOX inhibition affected ICH-associated physical damage of the brain tissue, we examined the effect of zileuton on the hematoma volume. Results of T2-weighted MRI images obtained at 72 h after ICH induction showed that zileuton had no effect on the hematoma volume (Fig. 2G, H). Despite the fact that zileuton did not reduce ICH-associated physical damage of the brain, daily treatment with this drug at either 3 or 10 mg/kg significantly ameliorated neurological functions of mice as assessed by the modified limb-placing test (Fig. 2I). Tendency for suppression of sensorimotor dysfunction by zileuton was observed also in the beam-walking test, although the difference from vehicle control did not reach statistical significance (Fig. 2J).

**Deletion of BLT1 inhibits neutrophil invasion and alleviates ICH-induced sensorimotor deficits.**

To obtain evidence for the involvement of LTB4 in the pathological events in ICH, we next examined the role of LTB4 receptors. Of two subtypes of LTB4 receptors, the high affinity receptor is designated as BLT1 and the other as BLT2. We found that mRNAs for both receptor subtypes were significantly increased after induction of ICH, where the increase in BLT1 mRNA (Fig. 3A) was more prominent than that in BLT2 mRNA (Fig. 3B). We particularly focused on BLT1, because BLT1 is expressed in inflammatory and immune cells including neutrophils and also because BLT1 mediates the chemotactic response of neutrophils (Yokomizo et al., 1997). BLT1-KO mice and their WT littermates received surgical procedures to induce ICH, and they were compared for the extent of neutrophil infiltration and sensorimotor dysfunction. The central region of the hematoma of BLT1-KO mice at 72 h after ICH contained a substantially decreased number of MPO-positive cells, as compared to that of WT mice (Fig. 3C, D). On the other hand, the absolute volume of the hematoma at 72 h did not show significant difference between WT mice and BLT1-KO mice (Fig. 3E, F). Importantly, BLT1-KO mice performed better than WT mice in both the modified limb-placing test (Fig. 3G) and the beam-walking test (Fig. 3H), and amelioration of the performance by BLT1 deletion reached statistical significance in the modified limb-placing test.
Therapeutic treatment of BLT antagonist inhibits neutrophil invasion and alleviates ICH-induced sensorimotor deficits. To address the validity of BLT₁ as a target of pharmacotherapy for ICH, we examined the consequences of pharmacological blockade of BLT₁ on the pathological events in ICH. In this set of experiments we used a BLT antagonist ONO-4057, which has originally been identified as an antagonist of LTB₄ receptor in human neutrophils that corresponds to BLT₁ (Kishikawa et al., 1992; Yokomizo et al., 1997). The drug has also been shown to exert therapeutic effects on several models of inflammatory diseases at an i.p. dose of 10 mg/kg (Saiwai et al., 2010) or at an oral dose of 100 mg/kg (Andoh et al., 2014). To evaluate whether post-treatment with the BLT antagonist could provide therapeutic effect, we administered ONO-4057 (30 and 100 mg/kg, p.o.) to mice once daily for 3 days starting from 3 h after induction of ICH. Treatment with ONO-4057 decreased the number of MPO-positive cells in the central region of the hematoma at 72 h after ICH, in a dose-dependent manner (Fig. 4A, B). On the other hand, the volume of the hematoma was not affected by either dose of ONO-4057 (Fig. 4C, D). As for sensorimotor functions, ONO-4057 at doses of both 30 and 100 mg/kg produced a beneficial effect as revealed by significant amelioration of the performance in the modified limb-placing test (Fig. 4E) and a significant decrease in the fault rate in the beam-walking test (Fig. 4F).

Therapeutic treatment of BLT antagonist suppresses ICH-induced inflammation and axon fragmentation. We further examined the effect of BLT antagonist on the inflammatory reactions and the axonal injury induced by ICH. Iba1 is a calcium binding protein whose expression is restricted to microglia/macrophages (Ohsawa et al., 2000). As expected, Iba1-immunopositive area was drastically increased in the perihematomal region at 72 h after ICH induction, as a result of the increased number of microglia/macrophages as well as the morphological changes of microglia into ameboid and swollen form (Fig. 5A). Treatment with ONO-4057 (100 mg/kg) partially prevented these changes (Fig. 5A). Quantification of the immunopositive signals in the perihematomal region revealed that ONO-4057 (100 mg/kg) tended to attenuate the increase of Iba1-immunopositive area (Fig. 5B). Moreover, anti-inflammatory effect of ONO-4057 was evident in the expression level of tumor necrosis factor α...
(TNF-α), a pro-inflammatory cytokine. That is, a robust increase in the expression of TNF-α mRNA was observed in the brain tissues at 72 h after ICH, whereas treatment with ONO-4057 inhibited this increase in a dose-dependent manner and the effect reached statistical significance at 100 mg/kg (Fig. 5C).

We also examined the structural integrity of axonal fibers in the internal capsule, because this brain region contains the cortico-spinal tract that conducts information for regulation of motor function (Ishida et al., 2011; Matsushita et al., 2013; Hijioka et al., 2016). Immunohistochemical examination of NF-H detected fibrous pattern of staining that reflected intact structures of the axonal fibers in the internal capsule of sham-operated mice, whereas NF-H immunoreactivity showed punctate appearance at 72 h after ICH that reflected destruction and fragmentation of the axonal structures (Fig. 5D). Results of the quantitative analysis of axon morphology as axonal shape index (Hijioka et al., 2016) indicated that deterioration of fibrous structures was partially but significantly attenuated by treatment with 100 mg/kg ONO-4057 (Fig. 5E).

Discussion

The present study was aimed to find out a novel therapeutic approach for ICH, with special reference to a neutrophil chemotactic factor LTB₄. We obtained evidence for a critical role of LTB₄ and its receptor BLT₁ in ICH associated with severe sensorimotor dysfunction.

Increased level of LTB₄ has been demonstrated in several central nervous system disorders such as ischemia (Namura et al., 1994) and subarachnoid hemorrhage (Shimizu et al., 1988), but the changes in the level of LTB₄ in ICH have not been addressed to date. In the present study we showed that the expression of mRNAs encoding 5-LOX and FLAP, the rate-limiting enzyme for LTB₄ biosynthesis and its activating protein, respectively, was upregulated at 24 h after induction of ICH. Correspondingly, LTB₄ content in the brain was also found to increase at 24 h after ICH induction. On the other hand, mRNA expression of CYP4F14, an LTB₄-metabolizing enzyme, was not upregulated until 48 h after induction of ICH, which may have allowed increase of LTB₄ at an earlier time point of 24 h. As we observed a delayed increase in
the expression of CYP4F14, whether LTB₄ metabolites are increased at later time points may deserve investigation to enable better understanding of the metabolic dynamics of LTB₄ in the brain after ICH. We should also note that LTA₄H (Rybina et al., 1997) and 5-LOX (Radmark et al., 2007) may undergo phosphorylation-dependent regulation of enzymatic activity, which may contribute to the changes in the production level of LTB₄ after ICH. Moreover, the cellular localization of 5-LOX and other factors and their changes in response to ICH are important points to be determined in further investigations. For example, 5-LOX may be derived in part from infiltrating neutrophils in the brain after ICH, whereas our preliminary observations suggest that neurons constitutively express 5-LOX (data not shown).

The observed effect of zileuton, a conventional and selective inhibitor of 5-LOX, is consistent with the idea that LTB₄ plays an important role in the pathogenic events in ICH. Several LOX inhibitors are known to have antioxidative properties, but zileuton is a weak antioxidant that scavenges free radicals and inhibits protein oxidation only at much higher concentrations than those required for 5-LOX inhibition (Czapski et al., 2012). Therefore, the biological effect of zileuton should be attributable to 5-LOX inhibition, although the contribution of antioxidative properties cannot be totally excluded. We confirmed that the tested doses of zileuton effectively abolished ICH-induced increase in LTB₄ in the brain. At the same doses, zileuton significantly diminished infiltration of neutrophils into the hematoma, suggesting that LTB₄ is indeed involved in neutrophil chemotaxis after ICH. In addition, zileuton partially improved the sensorimotor performance of mice after ICH, which suggests that inhibition of 5-LOX provides beneficial effect via suppression of LTB₄ production. A potential drawback of the usage of zileuton is that the drug inhibits production of all bioactive metabolites downstream of 5-LOX. Several of these metabolites such as lipoxin A₄ and resolvin E₁ have been known to suppress the chemotactic response of neutrophils (Papayianni et al., 1996; Arita et al., 2007). On the other hand, 5-LOX inhibition may also inhibit production of LTC₄ and LTD₄, and these cysteinyl leukotrienes reportedly contribute to neuronal injury and microglial activation under ischemic conditions (Zhang et al., 2013) and delayed vasospasm after subarachnoid hemorrhage (Kobayashi et al., 1992). Therefore, the net effect of 5-LOX inhibition might
obscure the contribution of LTB₄ to neutrophil infiltration and the resultant severity of the neurological symptoms in ICH.

Accordingly, we set our focus on LTB₄ receptors to address the role of LTB₄ in ICH pathology. BLT₁ is the high affinity LTB₄ receptor considered to mediate the chemotactic action of LTB₄ on neutrophils (Yokomizo et al., 1997). Consistent with this notion, BLT₁ mRNA increased in the brain after ICH, which seemed to be in parallel with the increase in the number of neutrophils, a major cell population that expressed BLT₁. Moreover, when ICH was induced in BLT₁-KO mice, the extent of sensorimotor dysfunction as well as the number of infiltrating neutrophils in the hematoma was substantially lower than that in WT mice. These results clearly indicate that BLT₁ stimulation plays an important role in the pathological consequences of ICH.

To address the validity of BLT₁ as a drug target for ICH therapy, we evaluated the effect of a BLT antagonist ONO-4057. As in the case with administration of zileuton or deletion of BLT₁ gene, treatment with ONO-4057 from 3 h after induction of ICH decreased the number of infiltrating neutrophils and ameliorated the sensorimotor performance of mice. Notably, the volume of hematoma was not affected either by zileuton, ONO-4057 or BLT₁ deletion, suggesting that BLT₁-targeted therapy can provide beneficial effect without diminishing physical damage of the brain tissues. It should be noted, however, that ONO-4057 is a non-selective BLT antagonist that may also block BLT₂-mediated cellular response such as LTB₄-induced lung contraction (Sakata et al., 2004). Although our results with BLT₁-KO mice strongly suggest pathogenic role of BLT₁ in ICH, contribution of BLT₂ blockade to the therapeutic action of ONO-4057 may deserve consideration. Another point to be taken into consideration is that BLT₁ is expressed in various inflammatory and immune cells including helper T cells (Yokomizo, 2011). Since infiltration of helper T cells has been observed in the brain of experimental ICH models (Mracsko et al., 2014), the influences on these cell populations might also contribute to the effects of deletion or blockade of BLT₁.

Theoretically, a potential advantage of BLT₁ over 5-LOX as a therapeutic target is that BLT₁ antagonism
does not interfere with the actions of lipid metabolites other than LTB₄, and therefore that the net effect of anti-inflammatory and neuroprotective actions should be more prominent than that in the case of 5-LOX inhibition. Indeed, we found that the activation of microglia/macrophages in the perihematoma region and the expression of TNF-α were reduced by ONO-4057. These effects were not evident in the case of 5-LOX inhibition by zileuton (data not shown). Although we did not address detailed mechanisms of the action of ONO-4057 on microglia/macrophages, they may involve direct actions of the drug on macrophages as well as indirect actions through inhibition of neutrophil infiltration because BLT₁ is not only in neutrophils but also in macrophages (Yokomizo, 2011). In any case, inhibition of the activation of microglia/macrophages is likely to contribute to alleviation of ICH-related dysfunction. In this context, minocycline as an inhibitor of microglial activation has been reported to produce therapeutic effects on experimental ICH model (Xue et al., 2010). On the other hand, we also demonstrated that ICH-induced fragmentation of the axon structures in the internal capsule was significantly reduced by ONO-4057. This effect may be attributable to the suppression of neutrophil infiltration, because a previous study that addressed the effect of neutrophil depletion demonstrated protection of the axonal function after ICH (Moxon-Emre and Schlichter, 2011). The internal capsule contains descending and ascending axon tracts that connect the cerebral cortex with the spinal cord. ICH-induced damage of these axon tracts may be a critical determinant of the severity of the neurological symptoms (Matsushita et al., 2013). Therefore, diminution of axon tract injury may have direct relation with the beneficial effect of ONO-4057 on the sensorimotor performance after ICH.

In the present study we observed only partial blockade of neutrophil infiltration even in the case with BLT₁ gene deletion, suggesting that neutrophil chemoattractants other than LTB₄ are also involved in ICH pathogenesis. In this context, expression of several chemokines such as CXCL1 and CXCL2 are upregulated in the brain in response to ICH, and we have previously demonstrated that an antagonist at chemokine receptors CXCR1/CXCR2 provides a partial therapeutic effect on experimental ICH model in mice (Matsushita et al. 2014). Moreover, pathogenic events in ICH involve multiple other aspects that
may proceed in a neutrophil-independent manner, such as cytotoxicity of thrombin and other proteases, and oxidative stress-related events by iron derived from heme degradation (Katsuki, 2010). All of these contributing factors may restrict the effectiveness of BLT₁ blockade as therapeutics for ICH. Nevertheless, the present findings indicate that LTB₄ is indeed involved in the promotion of neutrophil infiltration after ICH, and that the blockade of BLT₁ provides a significant, although not complete, therapeutic effect on ICH. Moreover, a previous microarray analysis on human ICH patients has demonstrated upregulation of 5-LOX and FLAP in the brain (Carmichael et al., 2008). Therefore, targeting LTB₄-BLT₁ axis is expected to produce therapeutic effect on ICH in humans, a disorder currently lacking effective means of pharmacotherapy.

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Authorship Contributions

Participated in research design: Hijioka, Kurauchi, Hisatsune, Seki, Koga, Yokomizo, Shimizu, Katsuki

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Footnotes

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Figure Legends

Fig. 1. ICH was accompanied by changes in the expression of the enzymes regulating LTB₄ production. (A-D) Expression levels of ALOX5 (A), ALOX5AP (B), LTA₄H (C) and CYP4F14 (D) mRNAs were quantified at indicated time after ICH induction. Data were normalized to GAPDH mRNA level in the same sample. The number of animals examined is indicated in each column. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the 0 h group. Data were analyzed by one-way ANOVA followed by Tukey’s multiple comparisons test (A, C, D) and non-parametrical Kruskal-Wallis test followed by Dunn’s multiple comparisons test (B).

Fig. 2. A 5-LOX inhibitor attenuated LTB₄ production, neutrophil invasion and sensorimotor dysfunction after ICH. (A-B) LTB₄ contents in the brain tissues were measured at 6 h (A) and 24 h (B) after induction of ICH with or without zileuton treatment. Zileuton was intravenously administered at 15 min, 24 h and 48 h after ICH induction. The number of animals examined is indicated in each column. *P < 0.05, **P < 0.01 by one-way ANOVA followed by Tukey’s multiple comparisons test. (C) ALOX5 mRNA level at 72 h after ICH induction. The number of animals is indicated in each column. ***P < 0.001 compared with the sham-operated group by one-way ANOVA followed by Tukey’s multiple comparisons test. (D) The time course of the changes in the number of MPO-positive cells in the central region of the hematoma. The number of samples examined is indicated in each column. **P < 0.01, ***P < 0.001 compared with 6 h group by one-way ANOVA followed by Tukey’s multiple comparisons test. (E) The effect of zileuton on the number of MPO-positive cells in the central region of the hematoma at 72 h after ICH induction. The number of animals is indicated in each column. *P < 0.05 compared with the vehicle-treated ICH group by one-way ANOVA followed by Tukey’s multiple comparisons test. (F) Representative images of MPO-positive cells in the central region of the hematoma at 72 h after ICH. Scale bars = 100 μm. (G) Representative T2-weighted nuclear magnetic resonance images at 72 h after ICH. Dashed lines indicate the edge of the hematoma. Scale bars = 2 mm. (H) The effect of zileuton on the hematoma volume. The
number of animals examined is indicated in each column. (I-J) Motor functions were evaluated by the modified limb-placing test (I) and the beam-walking test (J) at indicated time after ICH induction. The number of animals examined under each condition is given in parenthesis. *P < 0.05, **P < 0.01 compared with the vehicle-treated ICH group by two-way ANOVA with repeated measures followed by post hoc comparisons with the Bonferroni method.

Fig. 3. BLT₁-KO mice showed diminished neutrophil invasion and attenuated sensorimotor dysfunction after ICH. (A-B) Quantification of BLT₁ and BLT₂ mRNAs in the brain tissues of WT mice was conducted by qRT-PCR at indicated time after ICH. The number of animals examined is indicated in each column. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the 0 h group by one-way ANOVA followed by Tukey's multiple comparisons test. (C) Representative images of MPO-positive cells in the central region of the hematoma in WT or BLT₁-KO mice at 72 h after ICH. Scale bars = 100 µm. (D) The number of MPO-positive cells in the central region of the hematoma in WT or BLT₁-KO mice at 72 h after ICH. n = 7 for WT mice and 9 for KO mice. ***P < 0.001 compared with WT mice, by Mann-Whitney U-test. (E) Representative T2-weighted nuclear magnetic resonance images obtained at 72 h after ICH. Dashed lines indicate the edge of the hematoma. Scale bars = 2 mm. (F) The effect of BLT₁ deletion on hematoma volume. n = 7 for WT mice and 9 for KO mice. (G-H) Motor functions were evaluated by the modified limb-placing test (G) and the beam-walking test (H) at indicated time after ICH. n = 7 for KO sham mice, 9 for KO ICH mice, and 7 for WT ICH mice. *P < 0.05, **P < 0.01, ***P < 0.001 compared with ICH-induced WT mice, by two-way ANOVA with repeated measures followed by post hoc comparisons with the Bonferroni method.

Fig. 4. Blockade of LTB₄ receptor attenuated neutrophil invasion and sensorimotor deficits induced by ICH. (A) Representative images of MPO-positive cells in the central region of the hematoma at 72 h after ICH. Mice received oral administration of vehicle or ONO-4057 at 3, 27 and 51 h after ICH induction.
Scale bars = 100 µm. (B) The number of MPO-positive cells in the central region of the hematoma. The number of animals examined is indicated in each column. *P < 0.05 compared with the vehicle-treated ICH group by one-way ANOVA followed by Tukey's multiple comparisons test. (C) Representative T2-weighted nuclear magnetic resonance images obtained at 72 h after ICH. Scale bars = 2 mm. (D) The effect of ONO-4057 on the hematoma volume. The number of animals examined is indicated in each column. (E-F) Motor functions were evaluated by the modified limb-placing test (E) and the beam-walking test (F) at indicated time after ICH induction. The number of animals examined under each condition is given in parenthesis. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the vehicle-treated ICH group, by two-way ANOVA with repeated measures followed by post hoc comparisons with the Bonferroni method.

**Fig. 5.** Blockade of LTB4 receptor diminished inflammatory responses and axon injury induced by ICH. (A) Representative images of Iba1-positive cells in the perihematomal region at 72 h after ICH. Mice received oral administration of vehicle or ONO-4057 at 3, 27 and 51 h after collagenase injection. Dashed lines indicate the edge of the hematoma. Scale bars = 200 µm. (B) Quantitative results on the percentage of Iba1-immunopositive area within an image of 725 × 546 µm². The number of animals examined is indicated in each column. *P < 0.05, **P < 0.01, n.s., not significant compared with the sham-operated group by non-parametrical Kruskal-Wallis test followed by Dunn’s multiple comparisons test. (C) The expression level of TNF-α mRNA at 72 h after ICH. The number of animals examined is indicated in each column. *P < 0.05, ***P < 0.001 compared with the sham-operated group, #P < 0.05 compared with the vehicle-treated ICH group, by one-way ANOVA followed by Tukey's multiple comparisons test. (D) Representative images of NF-H-positive axons in the internal capsule within the hematoma at 72 h after ICH. Scale bars = 20 µm. (E) Quantitative results of the morphological changes of axonal fibers after ICH. The number of samples examined is indicated in each column. ***P < 0.001 compared with the sham-operated group, ####P < 0.001 compared with the vehicle-treated ICH group, by non-parametrical
Kruskal-Wallis test followed by Dunn’s multiple comparisons test.
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Fig. 2

A, B, and C: Bar graphs showing the levels of LTB₄ (ng/g tissue) and ALOX5 mRNA (Fold of sham) at 6 h, 24 h, and 3 days post-ICH induction, respectively, with and without Zileuton treatment. 

D: Graph showing the number of MPO-positive cells over time after ICH induction (h). 

E: Bar graph showing the number of MPO-positive cells at various doses of Zileuton. 

F: Images showing MPO staining in tissue sections from Sham + Vehicle and ICH + Vehicle groups. 

G: Images showing hematoma volumes for ICH + Vehicle, ICH + Zileuton (3 mg/kg), and ICH + Zileuton (10 mg/kg) groups. 

H: Bar graph showing hematoma volumes at different doses of Zileuton. 

I and J: Line graphs showing the score and fault rate over time after ICH induction, with and without Zileuton treatment.
Fig. 3

A

**BLT**

mRNA level (Fold of 0 h)

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<th>24</th>
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B

**BLT**

mRNA level (Fold of 0 h)

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<th>24</th>
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C

WT

BLT-KO

D

No. of MPO-positive cells

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E

WT

BLT-KO

F

Hematoma volume (mm³)

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G

Score

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H

Fault rate (%)

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</table>
Fig. 4

A

Sham + Vehicle  
ICH + Vehicle  
ICH + ONO (30 mg/kg)  
ICH + ONO (100 mg/kg)

B

No. of MPO-positive cells

ONO (mg/kg)  
0  
30  
100

ICH

C

Sham + Vehicle  
ICH + Vehicle  
ICH + ONO (30 mg/kg)  
ICH + ONO (100 mg/kg)

D

Hematoma volume (mm³)

ONO (mg/kg)  
0  
30  
100

ICH

E

Score

Time after ICH induction (h)

ICH + 0 mg/kg ONO (6)  
ICH + 30 mg/kg ONO (8)  
ICH + 100 mg/kg ONO (8)

F

Fault rate (%)

Time after ICH induction (h)

ICH + 0 mg/kg ONO (7)  
ICH + 30 mg/kg ONO (8)  
ICH + 100 mg/kg ONO (8)