Lipoxin A4 Reduces Inflammation Through Formyl Peptide Receptor 2/p38 MAPK Signaling Pathway in Subarachnoid Hemorrhage Rats

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Background and Purpose—Lipoxin A4 (LXA4) has been reported to reduce inflammation in several neurological injury models. We studied the effects of LXA4 on neuroinflammation after subarachnoid hemorrhage (SAH) in a rat model.

Methods—Two hundred and thirty-eight Sprague–Dawley male rats, weight 280–320 g, were used. Exogenous LXA4 (0.3 and 1.0 nmol) were injected intracerebroventricularly at 1.5 hours after SAH. Neurological scores, brain water content, and blood–brain barrier were evaluated at 24 hours after SAH; Morris water maze and T-maze tests were examined at 21 days after SAH. The expression of endogenous LXA4 and its receptor formyl peptide receptor 2 (FPR2), as well as p38, interleukin-1β, and interleukin-6 were studied either by ELISA or by Western blots. Neutrophil infiltration was observed by myeloperoxidase staining. FPR2 siRNA was used to knock down LXA4 receptor.

Results—The expression of endogenous LXA4 decreased, and the expression of FPR2 increased after SAH. Exogenous LXA4 decreased brain water content, reduced Evans blue extravasation, and improved neurological functions and improved the learning and memory ability after SAH. LXA4 reduced neutrophil infiltration and phosphorylation of p38, interleukin-1β, and interleukin-6. These effects of LXA4 were abolished by FPR2 siRNA.

Conclusions—Exogenous LXA4 inhibited inflammation by activating FPR2 and inhibiting p38 after SAH. LXA4 may serve as an alternative treatment to relieve early brain injury after SAH. (Stroke. 2016;47:00-00. DOI: 10.1161/STROKEAHA.115.011223.)

Key Words: blood–brain barrier • formyl peptide receptor 2 • inflammation • lipoxin A4 • subarachnoid hemorrhage

Subarachnoid hemorrhage (SAH) represents a subtype of stroke that carries a high mortality and disability.1 Early brain injury has been reported as the primary cause of mortality in SAH patients and has been considered a primary target for treatment.2 Recent studies have shown that anti-inflammation may attenuate early brain injury after experimental SAH.3,4 Lipoxin A4 (LXA4) is one of the important arachidonic acid metabolites and has potent anti-inflammatory properties mediated by its receptor formyl peptide receptor 2 (FPR2),5 including inhibiting pro-inflammatory cytokine production, suppressing the activities of metalloproteinases, and enhancing the clearance ability of macrophage.6–8 LXA4 exerted these biological functions through downregulating the activities of p38 mitogen-activated protein kinase (MAPK), which was mediated by FPR2.7,8,10 Several studies have focused on the neuroprotective effects of LXA4 after stroke. Administration of LXA4 methyl ester was reported to reduce proinflammatory cytokines tumor necrosis factor-α and interleukin (IL)-1β and upregulate anti-inflammatory cytokines IL-10 and transforming growth factor-β1 in the ischemic brain.11,12 However, the effects of LXA4 in early brain injury after SAH have not been investigated.

In the present work, we examined the role of LXA4 in a rat model of SAH. The time course of expression of LXA4, its receptor FPR2, and inflammation markers, as well as p38, were measured in the presence of exogenous LXA4.

Materials and Methods
All experiments were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

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SAH Animal Model and Experimental Protocol

Two hundred and forty male Sprague–Dawley rats weighing 280 to 320 g (Indianapolis) were used. The endovascular perforation model of SAH in rats was performed as reported previously. Briefly, rats were intubated transorally and mechanically ventilated throughout the operation period with 3% isoflurane anesthesia. A sharpened 4-0 monofilament nylon suture was inserted rostrally into the right internal carotid artery from the external carotid artery stump and perforated the bifurcation of the anterior and middle cerebral arteries. Sham-operated rats underwent the same procedures except the suture was withdrawn without puncture.

Two dosages (0.3 and 1.0 nmol) of exogenous LXA4 (Cayman Chemical Company) were injected intracerebroventricularly at 1.5 hours after SAH. SAH grades, neurological scores, and brain water content were measured at 24 hours. Water maze and T-maze were tested from 21 to 26 days and 27 days, respectively. LXA4 receptor FPR2 were knocked down by FPR2 siRNA (sc-40123; Santa Cruz Biotechnology) to determine the signaling pathway. All siRNAs were mixed with the same volume of transfection reagent (sc-29528; Santa Cruz Biotechnology). The expression and time course of LXA4 and FPR2 were examined by ELISA or Western blots both in cortex and hippocampus at 24 hours after SAH. The expression of LXA4 was also examined by ELISA after giving exogenous LXA4. The levels of ALX, p-p38 MAPK, IL-1β, and IL-6 were measured by Western blots at 24 hours after SAH.

Intracerebroventricular Drug Administration

Intracerebroventricular drug administration was performed as previously described. Rats were placed in a stereotaxic apparatus under 2.5% isoflurane anesthesia. The needle of a 10-μL Hamilton syringe (Microliter 701; Hamilton Company) was inserted through a burr hole into the right lateral ventricles at the following coordinates relative to bregma: 1.5 mm posterior, 1.0 mm lateral, and 3.2 mm below the horizontal plane of the skull. LXA4 (0.3 and 1.0 nmol) were injected at 1.5 hours after SAH by a pump at a rate of 0.5 μL/min, respectively. FPR2 siRNA (500 pmol/3 μL; Santa Cruz Biotechnology) or scrambled siRNA (500 pmol/3 μL; Santa Cruz Biotechnology) dissolved in transfection reagent (Santa Cruz Biotechnology) were injected at 24 hours before SAH induction by a pump at a rate of 0.5 μL/min. We have previously showed that siRNAs were successfully taken up by endothelial cells, ependymal cells, glial cells, and neurons in the brain after intracerebroventricular injection. To further confirm that siRNA can get to the brain region at the base of skull after intraventricular injection, a fluorescence conjugated siRNA-A (sc-36869; Santa Cruz Biotechnology) was injected 24 hours before SAH (n=2).

Severity of SAH

The severity of SAH was quantified by use of the previously published grading scale at the time of euthanasia. Briefly, animals were euthanized and the brains were removed. The basal cistern was divided into 6 segments. Each segment was allotted a grade from 0 to 3 depending on the amount of subarachnoid blood in the segment. The animals received a total score ranging from 0 to 18 after adding the scores from all 6 segments. Rats with mild SAH (SAH grades ≤7 at 24 hours) were excluded from the study.

Neurological Scores

Neurological scores were evaluated in a blinded fashion at 24 hours after SAH by a blinded observer according to the scoring system of Garcia et al with modifications.

Morris Water Maze

At day 21 to day 26 after SAH, Morris water maze was performed in a blinded setup as previously described. Briefly, it consists of 3 trials...
T-maze Test

T-maze test for spontaneous alternation has been used to examine exploratory behavior and working memory of hippocampus. As studied previously, rats were placed in the stem of a T-shaped maze and allowed to freely explore the 2 arms of the maze throughout a 10-trial continuous alternation session. The spontaneous alternation rate was expressed as the ratio of the alternating choices to the total number of the choice.

Brain Water Content

Brain water content was measured as previously described. Animals were euthanized, and the brains were removed at 24 hours after surgery. The brains were separated into left hemisphere, right hemisphere, cerebellum, and brain stem. Each part was weighed immediately after removal (wet weight). Brain specimens were dried in an oven at 105°C for 72 hours and weighed again (dry weight). The percentage of water content was calculated as ([wet weight−dry weight]/wet weight)×100%.

Blood–Brain Barrier Disruption

The permeability of blood–brain barrier (BBB) was evaluated on the basis of Evans blue extravasation, as described previously. The brain level of Evans blue was determined at 615 nm for spectrophotometric quantification.

ELISA for LXA4

The level of LXA4 was measured as previously described by a rat LXA4 ELISA kit (MyBioSource Inc) following the manufacturer’s instructions. The brain samples were collected at 24 hours after SAH and homogenized with 0.01 M phosphate-buffered saline. The supernatant was incubated together with LXA4-horseradish peroxidase (HRP) conjugate in precoated plate and then incubated with a substrate for HRP enzyme. Finally, the absorbance was measured spectrophotometrically at 450 nm in a microplate reader (Bio-Rad iMark).

Fluorescent Immunostaining

Double-fluorescence staining was performed at 24 hours after SAH as described previously. Sections were incubated overnight at 4°C with rabbit anti-FPR2 antibody (Santa Cruz Biotechnology), goat anti-ionized calcium-binding adaptor molecule 1 (Iba1), goat anti-glial fibrillary acidic protein (GFAP), and Neuronal nuclei (NeuN). B, Statistical analysis of the double-staining cells. The expression of FPR2 is low in brain in sham animals, and SAH increased its expression in microglias and astrocytes, but not in neurons at 24 h after SAH. n=3 for each group. *P<0.05 vs sham. Scale bar=30 μm.

Western Blotting

The brain samples were collected at 24 hours after SAH. Proteins of the ipsilateral cortex and hippocampus were extracted by homogenizing in RIPA buffer (Santa Cruz Biotechnology). Western blotting was performed as described previously using FPR2 (Santa Cruz Biotechnology), phospho-p38 (Santa Cruz Biotechnology), p38 (Santa Cruz Biotechnology), IL-1β (Abcam), and IL-6 (Santa Cruz Biotechnology).

Statistical Analysis

Data were expressed as the mean±SEM. Statistical differences among groups were analyzed by using 1-way ANOVA followed by Turkey post hoc test. Mortality was evaluated by Fisher’s exact test. P value of <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism for Windows.

Results

SAH Decreased the Level of LXA4 and Increased the Expression of Its Receptor FPR2: FPR2 Expressed in Microglias and Astrocytes, but Not in Neurons After SAH

After SAH, there were clear blood clots around basal cistern, and the SAH grades decreased gradually as time passed.
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There were significant differences of SAH grades between sham and SAH animals ≤ 72 hours (Figure I in the online-only Data Supplement; \( P < 0.05 \) between sham and SAH group), but no significant difference between SAH groups (Figure I in the online-only Data Supplement; \( P > 0.05 \) versus SAH groups).

The results of ELISA showed that the level of LXA4 both in cortex and hippocampus decreased from 6 hours after SAH and reached minimum at 24 hours (Figure 1A and 1B; \( P < 0.05 \) versus sham). Administration of LXA4 significantly increased the level of brain LXA4 both in sham and SAH animals at 24 hours after intracerebroventricular injection (Figure II in the online-only Data Supplement; \( P < 0.05 \) versus vehicle groups). However, the protein expression of FPR2 increased after SAH and peaked at 24 hours (Figure 1C and 1D; \( P < 0.05 \) versus sham). Double immunostaining of FPR2 with ionized calcium-binding adaptor molecule 1 (marker for microglia), glial fibrillary acidic protein (marker for astrocyte), and neuronal nuclei (marker for neuron) showed that FPR2 is intensely expressed and highly co-localized with ionized calcium-binding adaptor molecule 1 and astrocytes at 24 hours after SAH (Figure 2A and 2B; \( P < 0.05 \) versus sham). There was no colocalization of FPR2 with neuronal nuclei after SAH (Figure 2A and 2B; \( P > 0.05 \) versus sham).

Administration of LXA4 Decreased Brain Edema and Blood–Brain Barrier Permeability, Improved Neurological Functions, and Reduced Mortality at 24 Hours After SAH

Two dosages of LXA4 (0.3 and 1.0 nmol) were administrated intracerebroventricularly 1.5 hours after SAH. Brain water content, Evans blue extravasation, neurological scores, and mortality were measured at 24 hours after SAH. The data showed that LXA4 at 1.0 nmol decreased the brain water content (Figure 3A; \( P < 0.05 \) versus SAH+vehicle) and BBB permeability (Figure 3B; \( P < 0.05 \) versus SAH+vehicle), improved the neurological deficits (Figure 3C; \( P < 0.05 \) versus SAH+vehicle), and decreased the mortality (Figure 3D; \( P < 0.05 \) versus SAH+vehicle) at 24 hours after SAH. The results indicated that high dosage of LXA4 is an effective treatment for early brain injury, so we used this dosage to further determine the long-term effects and investigate the mechanism.

LXA4 Improved Spatial Learning and Memory Abilities 21 Days After SAH

Morris water maze was executed at 21–26 days after SAH. The results showed the animals in vehicle group had a significantly greater distance moved from the target (Figure 4A; \( P < 0.05 \) versus sham), need more time to reach the platform (Figure 4B; \( P < 0.05 \) versus sham), and spent less time in the probe quadrants.
Knockdown FPR2 by FPR siRNA Increased Brain Edema, Decreased Neurological Scores, and Aggravated Neutrophil Infiltration in LXA4-Treated Animals

To further investigate the potential mechanisms of LXA4, we administrated FPR2 siRNA or scramble siRNA with LXA4 treatment in SAH rats. We first showed siRNAs was transfected into neural cells in the brain region at the base of skull (Figure III in the online-only Data Supplement). FPR2 siRNA significantly increased brain water content (Figure 5A; \( P<0.05 \) versus SAH+LXA4+Scramble siRNA) and decreased the neurological scores (Figure 5B; \( P<0.05 \) versus SAH+LXA4+Scramble siRNA) when compared with scramble siRNA group at 24 hours after SAH. We also investigated the effects of LXA4 on neutrophil infiltration and measured the level of myeloperoxidase in brain cortex by immunostaining at 24 hours after SAH. Results from immunostaining indicated that LXA4 treatment significantly reduced the number of myeloperoxidase-positive cells in the cortex compared with vehicle group after SAH (Figure 5C and 5D; \( P<0.05 \) versus SAH+vehicle), and knockdown FPR2 reversed the effects of LXA4 (Figure 5C and 5D; \( P<0.05 \) versus SAH+LXA4+Scramble siRNA).

LXA4 Inhibited the Expression of IL-1\( \beta \) and IL-6 Dependent on FPR2/p38 Pathway

Administration of LXA4 had no significant effects on the protein level of its receptor FPR2 at 24 hours after SAH (Figure 6A; Figure IVA in the online-only Data Supplement; \( P>0.05 \) versus SAH+vehicle); however, administration of FPR2 siRNA knocked down the receptor efficiently (Figure 6A; Figure IVA in the online-only Data Supplement; \( P<0.05 \) versus SAH+LXA4+Scramble siRNA). LXA4 treatment inhibited the phosphorylation of p38 and the expression of IL-1\( \beta \) and IL-6 both in brain cortex (Figure 6B and 6D; \( P<0.05 \) versus SAH+vehicle) and in hippocampus (Figure IIIB and IIID in the online-only Data Supplement; \( P<0.05 \) versus SAH+vehicle) compared with vehicle group. Knockdown of FPR2 by FPR2 siRNA remarkably increased the expression of p38, IL-1\( \beta \), and IL-6 in LXA4-treated animals (Figure 6B and 6D; \( P<0.05 \) versus SAH+LXA4+Scramble siRNA and Figure IVB and IVD in the online-only Data Supplement; \( P<0.05 \) versus SAH+LXA4+Scramble siRNA).
Discussion

In this study, we investigated the role of LXA4 in anti-inflammation during early brain injury after SAH. Our data showed that high dosage of exogenous LXA4 reduced brain edema, preserved BBB integrity, improved neurological scores, as well as spatial learning and memory abilities in SAH rats; silence of its receptor FPR2 by FPR2 siRNA abolished the beneficial effects of LXA4; LXA4 ameliorated the outcome by suppressing neutrophil infiltration, inhibiting the expression of phosphorylated p38, IL-1β, and IL-6 both in the cortex and hippocampus. Our study confirmed that LXA4 provided neuroprotection in early brain injury through suppressing inflammation via FPR2/p38 signaling pathway. LXA4 might be a promising alternative treatment for SAH patients.

Previous studies have showed that LXA4 had anti-inflammatory actions in vivo when administered to the site of inflammation or systemically by intravenous injection, intraperitoneal injection, or oral route. However, BBB limits the access of therapeutic agents to the brain and presents a major challenge to the delivery of drugs. The molecular weight of LXA4 is 352.5 Da. It is little known whether LXA4 could cross the BBB. Delivery of LXA4 through intracerebroventricular injection has been reported to protect brain and reduce inflammation in focal cerebral ischemia reperfusion. So in the present study, we administrated LXA4 by intracerebroventricular injection.

LXA4 has been shown to exert its activity principally via the G protein–coupled lipoxin receptor FPR2. In immune system, it has been reported that LXA4 can decrease IgM and IgG production on activated human B cells through FPR2-dependent signaling, which downregulated NF-κB nuclear translocation. In addition, LXA4 suppressed the development of endometriosis through a mechanism that mediated was by FPR2. LXA4 significantly decreased airway inflammation and regulated the catabasis of eosinophilic inflammation by increasing NK cell–mediated eosinophil apoptosis and decreased IL-13 release by type 2 innate lymphoid cells, which was also mediated by FPR2. We found the protection of high dosage exogenous LXA4 could be blocked by FPR2 siRNA, which lead to increased brain edema and...

Figure 5. The protective effects of lipoxin A4 (LXA4) depended on formyl peptide receptor 2 (FPR2). FPR2 siRNA increased brain edema (A), deteriorated neurobehavioral deficits (B), and aggravated neutrophil infiltration (C and D) in LXA4-treated animals. n=6 for each group in A and B; n=3 for each group in C and D. *P<0.05 vs sham, †P<0.05 vs subarachnoid hemorrhage (SAH)+vehicle, and ‡P<0.05 vs SAH+LXA4.
BBB damage, and also deteriorated neurological impairments. These results suggested that the neuroprotective effects of LXA4 in SAH rats were mediated through its receptor FPR2, which is consistent with the previous reports.12,14,30

Inflammation is a key pathologic manifestation of early brain injury and is a crucial factor for poor outcome after SAH. Inflammation can cause BBB leakage, brain edema, and cell death, which result in neurological deficits and mortality. Microglia are the most active cells involved in regulating inflammatory processes in the brain, and astrocytes are also known to be directly involved in regulating inflammation after SAH.31 This study demonstrated that FPR2 co-localized with microglia and astrocytes after SAH, which suggested that microglia and astrocytes might be the targets of LXA4 in the brain. We further observed that the expression of FPR2 both in cortex and hippocampus increased after SAH, suggesting that SAH induces the activation of FPR2, and the activation of FPR2 was not enhanced by LXA4 treatment. Finally, we observed that FPR2 siRNA suppressed neutrophil infiltration and pro-inflammatory IL-1β and IL-6.

Recent studies have linked p38 MAPK to cytokine activation in response to inflammation after SAH.12,33 Our previous published data showed that significant increased phosphorylation of p38 MAPK was observed in brain after SAH.19 The activities of p38 MAPK could be downregulated by FPR2 receptor antagonist on endometriosis.7 It has been determined that p38 was activated in the arterial wall after SAH, leading to the development of vasospasm through the upregulation of inflammatory cytokines, which can be inhibited by p38-selective inhibitor.34 The IL-6 synthesis was inhibited by specific inhibitors of p38 MAPK in human astroglioma cells and primary rat astrocytes.35 In this study, p38 MAPK was phosphorylated, and the expression of IL-1β and IL-6 were increased after SAH; high dosage of LXA4 reduced the phosphorylation of p38 MAPK and inhibited the expression of IL-1β and IL-6. Based on these evidences, we suggest that LXA4 may inhibit the phosphorylation of p38 MAPK via FPR2 and resulted in decreased inflammatory response during early brain injury after SAH.

In conclusion, our observations indicated for the first time that LXA4 treatment reduced inflammation through FPR2/p38 MAPK signaling pathway in SAH rats. This study provided new information on inflammation after SAH, and LXA4 may be a future option for the treatment of SAH patients.
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Supplemental Figure I. SAH grades after SAH surgery. There were significant differences of SAH grades between sham and SAH animals up to 72 hours, but no significantly difference between SAH groups. n=6 for each group, *p<0.05 vs. Sham.
Supplemental Figure II. Administration of LXA4 significantly increased brain LXA4 in sham and SAH animals at 24 hours after LXA4 injection. n=6 for each group, *p<0.05 vs. Sham+vehicle, #p<0.05 vs. SAH+vehicle.
Supplemental Figure III. Results of siRNA transfection. In vivo transfected cells 24 h after intracerebroventricular injection of fluorescence conjugated Control siRNA-A in rats. Images were obtained from 20 μm cryosections of rat brain using an OLYMPUS BX51 microscope with fluorescence light. Scale bar=30μm.
Supplemental Figure IV. Administration of LXA4 reduced expression of IL-1β and IL-6 dependent on FPR2/p38 pathway in hippocampus. Administration of LXA4 had no effects on the expression of FPR2 after SAH (A), but increased the phosphorylation of p38 (B), and expression of IL-1β (C) and IL-6 (D). Silencing FPR2 by siRNA significantly decrease the level of FPR2 and removed the effects of LXA4. n=6 for each group. #p<0.05 vs. SAH+vehicle, &p<0.05 vs. SAH+LXA4, %p<0.05 vs. SAH+LXA4+ sramble siRNA.