Toll-Like Receptor 4 Mediates Retinal Ischemia/Reperfusion Injury Through Nuclear Factor-κB and Spleen Tyrosine Kinase Activation

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Purpose. Inflammatory response has a critical role in neuronal damage after retinal ischemia/reperfusion (I/R) injury, and is regulated tightly by the toll-like receptor (TLR) 4. This study aimed to determine whether TLR4 is involved with injury in an ocular ischemic syndrome mice model and to clarify the downstream pathway of TLR4.

Methods. To cause retinal ischemia, we ligated the unilateral external carotid artery and the pterygopalatine artery of mice for 5 hours. Five days after reperfusion, retinal histologic analysis was performed. To examine the downstream pathway of TLR4, we analyzed the changes in phosphorylation of nuclear factor-κB (NF-κB) by Western blotting. In addition, we evaluated the expression of phosphorylated spleen tyrosine kinase (Syk), which is an adaptor protein of TLR4, and the effects of a Syk inhibitor (piceatannol) against the retinal ischemic damage and TLR4 signaling.

Results. TLR4 knock-out (KO) mice significantly inhibited the histologic damage induced by I/R compared to wild-type mice. The expression of TLR4 was upregulated after I/R in wild-type mice. The phosphorylation level of NF-κB after I/R in TLR4 KO mice was decreased compared to that in wild-type mice. The phosphorylated Syk expression was upregulated after I/R, and the upregulation was inhibited in TLR4 KO mice. Piceatannol inhibited the histologic and functional retinal damage, and reduced the phosphorylation level of NF-κB induced by I/R.

Conclusions. These data indicate that TLR4 has a pivotal role in the pathogenesis of ocular ischemic syndrome, and Syk and NF-κB are key molecules in TLR4 signaling in retinal ischemia.

Keywords: Toll-like receptor 4, nuclear factor-κB, spleen tyrosine kinase, piceatannol

Retinal ischemia, which leads to irreversible neuronal cell death, is the cause of many ocular diseases, such as diabetic retinopathy, rubebiotic glaucoma, and ocular ischemic syndrome. Ocular ischemic syndrome is the generic term given to a variable spectrum of aggregated ocular signs and symptoms that result from chronic ocular hypoperfusion. Ocular ischemic syndrome is usually secondary to severe carotid artery occlusion, and it may involve amaurosis fugax, central retinal artery occlusion, and branch retinal artery occlusion. To date, the pathologic mechanism of ocular ischemic syndrome remains poorly understood. Recently, we reported a new retinal ischemic mouse model in which the external carotid artery (ECA) and the pterygopalatine artery (PPA) were ligated.1 This animal model is useful for the clarification of pathologic mechanisms underlying the ocular ischemic syndrome.

Toll-like receptors (TLRs) are pattern recognition receptors that are key players of the innate immune response and are thought to have an important role in the central nervous system response to the injury-induced endogenous ligands termed as damage-associated molecular patterns as well as toward pathogens.2 TLRs are likely candidates for the initiation of inflammation after ischemia/reperfusion (I/R). In particular, we found that TLR4, but not TLR3 or TLR9, has a pivotal role in the pathogenesis of cerebral ischemic damage,3 and TLR4–NADPH oxidase 4 (NOX4) signal-mediated ROS production might contribute to the damage.4 TLR4 is expressed in the retina,5,6 and TLR4-deficient mice inhibited high IOP-induced ischemic neuronal cell death and increase of proinflammatory gene expression.7 These findings suggest that TLR4 has a crucial role in retinal ischemic injury. However, the downstream pathway of TLR4 in ischemic retinal injury still is not clearly understood. Although nuclear factor-κB (NF-κB) is known as a transcriptional factor of TLR4 signaling in retinal ischemia,7 other signaling molecules remain to be discovered.

The aim of our study was to determine whether TLR4 is involved with injury in the ocular ischemic syndrome mice model and to clarify the downstream pathway of TLR4.

Materials and Methods

Animals
All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision
Research, and they were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. A randomized, blind study was conducted, and efforts were made to minimize suffering and the number of animals used in the study. Pharmacologic experiments were performed using male ddY mice (n = 38; Japan SL C-ICR, Hamamatsu, Japan). TLR4 knock-out (KO) mice were obtained from Shizuo Akira and Satoshi Uematsu (n = 40; Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan), and backcrossed with C57BL/6J for 9 interbreeding generations. The C57BL/6J mice (n = 84) were used as control. The animals were housed at 23°C ± 3°C, under 12-hour light/dark cycles (lights on from 8 AM-8 PM). Each animal was used only for one experiment.

**Retinal Ischemia Model in Mice**

Anesthesia was induced using 2.0% to 3.0% isoflurane and maintained with 1.0% to 1.5% isoflurane (both in 70% N2O/30% O2), which was delivered using an animal anesthesia machine (Sof Lander; Sin-ei Industry Co. Ltd., Saitama, Japan). Body temperature was maintained at 37.0°C to 37.5°C with the aid of a temperature control system (NS-TC 10; Neuroscience, Inc., Tokyo, Japan). Retinal ischemia was induced by ECA and PPA ligations, as described in our previous report. Briefly, after a midline skin incision, the left common carotid artery was exposed, and the ECA was ligated. The internal carotid artery and its first branch were dissected, and the PPA was ligated. Ischemia was maintained for 5 hours, after which the ligatures were removed. All of the above procedures were performed while the animal was under anesthesia.

**Histology**

Mice were euthanized under anesthesia. Left eyes were enucleated and kept immersed in a fixative solution containing 4% paraformaldehyde for at least 24 hours at 4°C. Eight paraffin-embedded sections (5 μm thickness) were cut through the optic disc, parallel to the maximum circumference of the eyeball. After extraction of the eyeball, the tissue was homogenized in cell-lysis buffer by using a Physcotron homogenizer (Microtec Co., Ltd., Chiba, Japan). The lysate was centrifuged at 12,000 rpm for 20 minutes, and the supernatant was used for subsequent experiments. Protein concentrations were measured by comparison with a known concentration of BSA by using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). A mixture of equal parts of protein aliquot and sample buffer with 10% 2-mercaptoethanol was subjected to 15% SDS-PAGE. The separated protein was then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA). Transfers were blocked for 30 minutes at room temperature with 5% Block One-P (Nacalai Tesque, Inc., Kyoto, Japan) in 10 mM Tris-buffered saline containing 0.05% Tween 20, and then incubated overnight at 4°C with the primary antibody. For immunoblotting, the following primary antibodies were used: phosphorylated-JNK (Thr185/Tyr185) rabbit polyclonal antibody (1:1000; Cell Signaling, Danvers, MA), phosphorylated-p38 (Thr180/Tyr182) rabbit polyclonal antibody (1:2000; Promega, Tokyo, Japan), phosphorylated-c-Jun (Ser73) rabbit monoclonal antibody (1:1000; Cell Signaling), phosphorylated-NF-κB (Ser536) rabbit polyclonal antibody (1:1000; Cell Signaling), phosphorylated-spleen tyrosine kinase (Syk; Tyr323) rabbit polyclonal antibody (1:1000; Cell Signaling), TLR4 mouse monoclonal antibody (1:1000; IMAGE-NEX, San Diego, CA), JNK rabbit polyclonal antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), p38 rabbit polyclonal antibody (1:1000; Cell Signaling), c-Jun rabbit monoclonal antibody (1:1000; Cell Signaling), NF-κB rabbit polyclonal antibody (1:1000; Cell Signaling), Syk rabbit polyclonal antibody (1:1000; Cell Signaling), and β-actin mouse monoclonal antibody (1:5000; Sigma-Aldrich, St. Louis, MO). Goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG (1:2000) was used as a secondary antibody. The immunoreactive bands were visualized using Immuno Star LD (Wako Pure Chemical, Osaka, Japan), and then measured using LAS-4000 Mini (Fuji Film Co., Ltd., Tokyo, Japan).

**Immunohistochemistry**

The eyes were enucleated, fixed in 4% paraformaldehyde 24 hours at 4°C, immersed in 25% sucrose for 48 hours at 4°C, and embedded in optimal cutting temperature compound (Sakura Finetechanical Co., Ltd., Tokyo, Japan). Transverse cryostat sections of 10 μm thickness were cut and placed on slides (MAS COAT; Matsunami Glass Ind., Ltd., Osaka, Japan). The retinal sections were blocked in nonimmune serum from species of secondary antibody and incubated with primary antibody for overnight 4°C. Then, they were washed with PBS and incubated secondary antibody for 1 hour. Finally, they were counterstained by Hoechst 33342, and mounted in Fluoromount (Diagnostic BioSystems, Pleasanton, CA). For immunostaining, the following primary antibodies were used: TLR4 mouse monoclonal antibody (1:100; IMAGE-NEX), and they were incubated overnight at 4°C. Alexa Fluor 488 F(ab)2 fragment of goat anti-mouse IgG (H+L) antibody (1:1000; Invitrogen, Carlsbad, CA) was used as a secondary antibody, and incubated for 1 hour at room temperature. The sections were observed under a confocal microscope (FLUOVIEW FV10i; Olympus, Tokyo, Japan).

**Drug Treatment**

Piceatannol was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). It was suspended in distilled water containing 0.5% sodium carboxymethyl cellulose (CMC) immediately before use. Mice were administered piceatannol intraperitoneally at a dose of 10 or 50 mg/kg, or an identical volume (see below) of CMC. Each animal received either treatment at 30 minutes before ischemia, just after reperfusion, at 7 hours after the start of reperfusion, and then once a day for the next 4 days. The injection volume was adjusted to 10 mL/kg body weight.

**ERG Recording**

ERG recordings were performed as described in our previous report, at 5 days after I/R. Scotopic ERG records were used to
evaluate retinal function in mice that had been kept in a completely dark room for 24 hours. They were anesthetized intraperitoneally with a mixture of ketamine (120 mg/kg; Daiichi-Sankyo, Tokyo, Japan) and xylazine (6 mg/kg; Bayer Health Care, Tokyo, Japan), and their pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Santen Pharmaceutical Co., Ltd., Osaka, Japan). Flash ERG was recorded in the left eye of each dark-adapted mouse by placing a golden-ring electrode (Mayo, Aichi, Japan) in contact with the cornea and a reference electrode (Nihon Kohden, Tokyo, Japan) through the tongue. A neutral electrode (Nihon Kohden) was inserted subcutaneously near the tail. All procedures were performed in dim red light, and the mice were kept warm throughout the procedure. The amplitude of the a-wave was measured from the baseline to the highest a-wave peak (the lowest peak of curves), and the b-wave was measured from the highest a-wave peak to the highest b-wave peak (the highest curves).

**Statistical Analysis**

Data are presented as the mean ± SEM. Statistical comparisons were made using a 2-tailed Student’s *t*-test or Dunnett’s test by using STATVIEW version 5.0 (SAS Institute, Cary, NC). *P* < 0.05 was considered to indicate statistical significance.

**RESULTS**

**Effect of TLR4 KO Against Ischemia/Reperfusion-Induced Retinal Damage**

We observed loss of cells in the GCL, and a decrease in the thickness of the INL and IPL in both wild-type (WT) and TLR4 KO mice retinas at 5 days after I/R. However, in contrast with the WT mice, TLR4 KO mice exhibited a significantly higher

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survival rate of cells in the GCL and improvement of thickness of INL (Figs. 1B, 1C). Although the IPL thickness was expected to remain normal in TLR4 KO mice, significant difference was not observed ($P = 0.148$) when compared to WT after I/R (Fig. 1D). 

**TLR4 Expression and Localization in the Mouse Retina Following Ischemia/Reperfusion**

We investigated changes in TLR4 expression in the retinal tissue at 5 hours after ligation of arteries, and at 1 and 19 hours after I/R in C57BL/6J mice by Western blot analysis. TLR4 protein expression levels tended to increase at 5 hours after ischemia compared to the control group, and they increased significantly at 1 and 19 hours after I/R (Figs. 2A, 2B).

Moreover, in an immunohistochemistry study, we also confirmed upregulation of TLR4 in the inner retina at 1 or 19 hours after I/R (Fig. 2C). To identify the localization in the activation of TLR4 in the ischemic retina, we performed double-immunostaining with TLR4 and retinal markers. Brn-3a is a well-known marker of retinal ganglion cells (RGC), and GFAP is known as a marker of astrocyte. TLR4 or Brn-3a did not merge before and after I/R. However, after I/R, TLR4 was expressed strongly around the RGCs (Supplementary Fig. S1A). Moreover, TLR4 or GFAP did not merge after I/R (Supplementary Fig. S1B).

**Downstream Signaling Pathway of TLR4**

To investigate the signaling pathway of TLR4 in retinal ischemia, we examined the phosphorylation of NF-kB, p38, JNK, and c-Jun. The ratio of phosphorylated NF-kB to total NF-kB was increased significantly in retinal extracts of WT mice (versus control mice) at 24 hours after the onset of ischemia in WT mice (Figs. 3A, 3B). The activation of NF-kB was observed; however, its activation was downregulated compared to 19 hours after I/R, and may be returned to the baseline in WT and TLR4 KO mice 5 days after retinal I/R (Supplementary Figs. S2A, S2B). The levels of phosphorylated-JNK, phosphorylated-p38, and phosphorylated-c-Jun were increased markedly in retinas of WT mice (versus control mice) at 12 hours after the onset of ischemia (expressed relative to total JNK, total p38, and total c-Jun, respectively) in WT mice (Figs. 3C–H). In TLR4 KO mouse
retina, I/R-induced phosphorylation of NF-κB was reduced significantly compared to that in WT mouse retina following I/R (Figs. 3A, 3B), but JNK, p38, and c-Jun levels were not significantly different in retinas of TLR4 KO mice and WT mice (Figs. 3C–H).

Expression Level of Syk and Phosphorylated-Syk in Mouse Retina Following Ischemia/Reperfusion

To analyze further the downstream signaling pathway of TLR4, we analyzed the protein expression levels of total Syk and phosphorylated-Syk in the retinas of WT mice and TLR4 KO mice.
mice after I/R by Western blot analysis. In WT C57BL/6J mouse retina, the expression levels of phosphorylated-Syk and total Syk were increased significantly at 19 hours after I/R, compared to the control mice (Figs. 4A–C). At 19 hours after I/R, increase of phosphorylated-Syk and total Syk were reduced significantly in the retina of TLR4 KO mice when compared to WT mice (Figs. 4D–F). The activation and expression of Syk were not significant, and may be returned to baseline in WT and TLR4 KO mice at 5 days after retinal I/R (Supplementary Figs. S2C–E).

**Effect of a Syk Inhibitor Against Retinal Damage and Activation of NF-κB**

To study the association between Syk and retinal ischemic damage, we evaluated the effects of a Syk inhibitor, piceatannol, against functional and histologic damage induced by I/R. Intraperitoneal administration of piceatannol, a Syk inhibitor, inhibited an increased phosphorylation of Syk after I/R (Supplementary Fig. S3). The functional consequences of piceatannol were assessed by recording the ERG response (Figs. 5A–C). In the control (no I/R) group, a-wave and b-wave amplitudes were increased in a light intensity-dependent manner. Each of these amplitudes was reduced significantly in the vehicle-treated group compared to the control mice at day 5 after I/R. The piceatannol-treated (50 mg/kg) group exhibited significantly less reduction in the b-wave amplitudes compared to the vehicle-treated group (Figs. 5A–C). For histologic evaluation, representative images of retinal histology were taken at 5 days after I/R (Fig. 5D). I/R caused a decrease in the cell number of GCL, and a decrease in the thickness of the IPL and INL in the retina of I/R plus vehicle-treated mice compared to that of control mice at 5 days after I/R. Administration of piceatannol (50 mg/kg) significantly prevented such reductions in the GCL cell number, and maintained the thickness of IPL and INL compared to the vehicle group (Figs. 5E–G).
FIGURE 5. Effect of a Syk inhibitor against retinal damage and activation of NF-κB. (A) Representative ERG recordings from retinas treated with vehicle, I/R plus vehicle, or I/R plus piceatannol (50 mg/kg). Stimulus flashes were delivered at 0.98 log cd/m². (B, C) Intensity-response functions for dark-adapted (B) a-wave and (C) b-wave amplitudes. (D) Representative photographs (hematoxylin and eosin-stained retinal sections) showing retinas treated with (a) vehicle, (b) I/R plus vehicle, or (c) I/R plus piceatannol (50 mg/kg). (E-G) Quantitative analysis of the cell number in GCL.
To investigate the relationship between Syk and the TLR4-NF-κB pathway, we examined the level of phosphorylation in NF-κB. In piceatannol-treated (50 mg/kg) mice, I/R-induced phosphorylation of NF-κB was reduced significantly compared to WT mice exposed to I/R (Figs. 5H, 5I).

**DISCUSSION**

In our study, TLR4 expression was upregulated after I/R injury. TLR4 KO mice reduced the retinal damage after I/R, and inhibited the increase of phosphorylated NF-κB and Syk after I/R. Furthermore, the Syk inhibitor, piceatannol, protected the retina from I/R-induced injury and inhibited NF-κB phosphorylation (Fig. 6).

Previous reports suggest that TLR4 participates in retinal and cerebral ischemic injury, which were induced by increasing the IOP and middle cerebral artery occlusion, respectively.7,11,12 Indeed, the expression of TLR4 was upregulated around the RGCs, which were damaged after I/R. These findings indicated that TLR4 is involved in the retinal damage induced by I/R.

The neuroprotection observed in TLR4 KO mice raises the following question: which molecule is activated by TLR4, and what leads to cell death in retinal ischemia? NF-κB and activator protein 1 (AP-1) are known as transcriptional factors of TLR4 signaling, which induce proinflammatory cytokine production. In unstimulated cells, NF-κB is retained in the cytoplasm by its inhibitory protein, IκB.13 When TLR4-NF-κB signaling is stimulated, TNF receptor associated factor (TRAF) 6 induces the phosphorylation of IκB, targeting IκB for rapid degradation through a ubiquitin-proteasome pathway, in turn releasing NF-κB and allowing its entry into the nucleus, where it regulates gene expression.14 Serine residue 536 of NF-κB p65 is the phosphorylation site in the p65 transactivation domain and its phosphorylation is required for NF-κB activation.15 In our study, increase in the phosphorylation level of Ser536 of NF-κB p65 following I/R was suppressed in TLR4 KO mice. This finding indicated that the suppression of NF-κB activation is caused by TLR4 deficiency. Another transcriptional factor, AP-1, is a dimeric basic leucine zipper (bZIP) protein, and it comprises members of the Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) families. Among these AP-1 family proteins, c-Jun is thought to have a central role in inflammatory responses. Moreover, AP-1 activation in TLR signaling is mediated by MAP kinases, such as JNK and p38.16

In our study, although the phosphorylation levels of c-Jun, JNK, and p38 were upregulated significantly after I/R, these levels were not significantly different between WT and TLR4 KO mice. In a previous report, JNK and p38 were reported to be activated by various stresses, such as oxidative stress, but not by TLR4 signaling. JNK and p38 also were reported to be involved in apoptosis.17 These findings indicated that the activation of TLR4 signaling pathway in retinal I/R leads to the activation of NF-κB (Fig. 6), but not AP-1.

Syk is an ubiquitous nonreceptor tyrosine kinase, and it has a central role in immunoreceptor signaling involving, Dectin-1, Mincl, and presumably TLR4 signaling.18 Previous experiments have demonstrated that TLR4 coimmunoprecipitates with Syk in neutrophils, monocytes, and macrophages, even in the absence of any stimuli.19–21 Upon monocyte and neutrophil stimulation with lipopolysaccharide (LPS) and macro-

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**Figure 6.** Putative mechanism for TLR4 signaling in retinal ischemia. TLR4 is activated in response to damage-associated molecular patterns (DAMPs), and then Syk is phosphorylated. TLR4-Syk pathway leads to proinflammatory gene expression via NF-κB phosphorylation, resulting in retinal cell death.
phage stimulation with minimally modified LDL, Syk phosphorylation was increased. 19–22 Additionally, Syk expression was more prominent in intestinal ischemia. 23 In our study, phosphorylated-Syk and total Syk were upregulated in the retina after I/R. Moreover, piceatannol, a Syk-selective inhibitor that suppressed phosphorylation of Syk, 24 decreased functionally and histologic damages in the retina that were induced by I/R. Furthermore, we observed that I/R-induced upregulation of phosphorylated-Syk and total Syk were suppressed in TLR4 KO mice. Therefore, these upregulations were found to be TLR4-dependent in ischemic retinal injury. These findings suggested that Syk is involved in retinal damage induced by I/R, and that TLR4 mediates Syk phosphorylation and expression (Fig. 6). The role of Syk in TLR4 signaling during retinal ischemia is not yet clear and remains to be elucidated. To confirm the role of Syk in TLR4–NF-κB signaling, we evaluated NF-κB phosphorylation in piceatannol-treated mouse retina after I/R. Similar to the evaluation of I/R-induced NF-κB phosphorylation between WT mice and TLR4 KO mice, piceatannol also suppressed the increase of NF-κB p65 (Ser536) phosphorylation after I/R. This result is similar to a previous report 25 that another Syk inhibitor, Sc-WE, suppressed MyD88-dependent NF-κB activation induced by LPS in HEK293 cells. Additionally, piceatannol inhibited LPS-induced NF-κB activation, whereas it did not affect the activation of p38 and JNK in RAW 264.7 cells. 26 Sc-WE also diminished the NF-κB-mediated luciferase activity in LPS-treated RAW 264.7 cells, whereas the AP-1-mediated pathway remained unaffected. 25 Therefore, it can be concluded that Syk is involved in TLR4–NF-κB signaling (Fig. 6).

In conclusion, we demonstrated that TLR4 deficiency in mice exhibited tolerance to retinal damage and NF-κB phosphorylation following I/R. Piceatannol also inhibited the retinal damage and NF-κB phosphorylation induced by I/R. Taken together, the data indicated that TLR4 signaling is involved in degenerative diseases of the retina, such as ocular ischemic syndrome, and Syk and NF-κB are key molecules in TLR4 signaling in retinal ischemia.

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