Optic neuritis refers to inflammation of the optic nerve, with retinal ganglion cell (RGC) loss. An epidemiologic study in the United States estimated that the annual incidence rate of optic neuritis was 5 per 100,000 population. There is a strong association between optic neuritis and multiple sclerosis (MS), an acute inflammatory demyelinating syndrome of the central nervous system (CNS) characterized by progressive immune-mediated destruction of the myelin sheath and accumulated neurological disability. Optic neuritis can result in severe, irreversible visual loss, especially the optic-spinal form of MS or neuromyelitis optica. Thus, much attention is focused on finding a treatment for this disease that can restore visual function.

The major role of the renin-angiotensin system (RAS) in the cardiovascular system is well known. Renin, a proteolytic enzyme primarily released by the kidneys, cleaves angiotensinogen to angiotensin I (Ang I). Ang I is further processed by angiotensin-converting enzymes (ACEs), including ACE2, to different angiotensin cleavage products. Among these, angiotensin II (Ang II) is the principal effector molecule of the RAS, acting on its target cells, mainly via the Ang II type 1 receptor (AT1R). Currently, Ang II receptor antagonists and ACE inhibitors are used as prescribed drugs to treat high blood pressure. When components of the RAS, such as ACEs and Ang II, were expressed in the immune system in experimental autoimmune encephalomyelitis (EAE), an experimental animal model of multiple sclerosis, inhibition of these components produced in different steps of the RAS system all markedly ameliorated the course of EAE. Moreover, Ang II has been identified as a potential therapeutic target for neurodegeneration in neuroinflammatory diseases.
paracrine mediator, which sustains inflammation in the CNS by up-regulating transforming growth factor (TGF)-β.\textsuperscript{13} AT1Rs were reported to be primarily expressed in CNS-resident cells during EAE.\textsuperscript{13} In vitro, astrocytes and microglia responded to Ang II treatment by inducing TGF-β expression via a pathway involving TGF-β–activating protease thrombospondin-1.\textsuperscript{13} When TGF-β up-regulation in astrocytes and microglia during EAE was blocked using candesartan, an inhibitor of AT1R, paralysis and blunted lymphocyte infiltration into the CNS were ameliorated.\textsuperscript{13} Thus, the RAS may be a potential new target for optic neuritis therapy.

Toll-like receptors (TLRs) are expressed in many cell types, and their expression levels are altered under pathologic conditions.\textsuperscript{14–16} For example, the expression of TLR3 and TLR4 was up-regulated in patients with MS, and the expression of TLR2, TLR4, and TLR9 was up-regulated in the spinal cord of mice with EAE.\textsuperscript{17,18} Consistent with these reports, in vivo treatment with specific TLR ligands exacerbated EAE, whereas targeted disruption of selected TLRs prevented EAE.\textsuperscript{17,19–21} These results suggest that TLRs have critical roles in MS and EAE.\textsuperscript{22} However, the molecular mechanisms that regulate TLRs during inflammatory autoimmune diseases remain elusive. One study reported that Ang II up-regulated TLR4 mRNA and protein expression in mouse mesangial cells and that this effect was mediated through AT1R.\textsuperscript{23} We previously reported that Ang II stimulated TLR4 and lipopolysaccharide (LPS)–induced production of inducible nitric oxide synthase (iNOS) in cultured retinal Müller glial cells and that this effect was suppressed by candesartan.\textsuperscript{24} Moreover, candesartan exerted neuroprotective effects on RGC loss in a mouse model of normal tension glaucoma by suppressing TLR4 and LPS-induced iNOS expression in the retina.\textsuperscript{24} The potential role of Ang II in modulating the expression of TLRs during EAE remains to be explored. In the present study, we investigated the effects of candesartan on optic neuritis and demonstrated an association between the RAS and innate immunity during EAE.

Materials and Methods

Animals

Female C57 BL/6J mice (CLEA Japan, Tokyo, Japan) were used in the experiments in accordance with the Tokyo Metropolitan Institute of Medical Science Guidelines for the Care and Use of Animals. The mice were aged 6 to 8 weeks at the time of immunization. All the experiments were approved by the Tokyo Metropolitan Institute of Medical Science.

Reagents

CV-11974 and its prodrug candesartan cilexetil were gifts from Takeda Pharmaceutical Co., Ltd., Osaka, Japan.\textsuperscript{25} CV-11974 was used for in vitro assays, and candesartan cilexetil was used for in vivo experiments. For the sake of simplicity, throughout the article, candesartan refers to candesartan cilexetil. Ang II was purchased from Sigma (St. Louis, MO), and SN50 was purchased from Calbiochem (Rotkreuz, Switzerland).

Induction of EAE, Administration of Candesartan, and Clinical Scoring

All the experiments were conducted according to the experimental timeline summarized in Figure 1, A and B. EAE was induced in mice using the myelin oligodendrocyte glycoprotein (MOG)\textsubscript{35–55} peptide (MEVG-WYRSPFSRVVHLRNYGK), as previously reported.\textsuperscript{26} Briefly, the mice received s.c. injections with 100 μg of MOG\textsubscript{35–55} mixed with 500 μg of heat-killed Mycobacterium tuberculosis H37RA (Difco, Schwechat, Austria) emulsified in complete Freund’s adjuvant. Each mouse also received i.p. injections of 500 ng of pertussis toxin (Seikagaku, Tokyo, Japan) immediately and 48 hours after immunization. The mice were divided into two groups (Figure 1, A and B). In the blood sampling group (Figure 1A), to collect enough blood for measuring the concentrations of Ang II using an enzyme-linked immunosorbent assay, the mice were anesthetized with sodium thiopental, the chest cavity was opened, and blood was drawn directly from the heart on day 7, 10, and 25 after MOG immunization. The mice were euthanized immediately after the procedure. Serum Ang II concentrations were measured using an enzyme-linked immunosorbent assay kit (E90005Mu, Uscn Life Science Inc., Wuhan, China). In the treatment group (Figure 1B), MOG-immunized mice were treated with candesartan (10 mg/kg in 0.5% carboxymethyl cellulose) or vehicle (0.5% carboxymethyl cellulose) by oral gavage once daily throughout the whole experimental period. Clinical signs were scored daily as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, flaccid tail; 3, impairment of righting reflex; 4, partial hind limb paralysis; 5, complete hind limb paralysis; 6, partial body paralysis; 7, partial forelimb paralysis; 8, complete forelimb paralysis or moribund; and 9, death.

Histologic Analysis

On day 25 after immunization, the mice were perfused with Zamboni’s fixative (2% paraformaldehyde and 15% picric acid in 0.1 mol/L phosphate buffer, Sigma), and eyes, optic nerves, and lumbar spinal cords were removed.\textsuperscript{27,28} The eyes were postfixed in 3% glutaraldehyde solution (3% glutaraldehyde, 9% formaldehyde, 37.5% ethanol, and 12.5% acetic acid in distilled water) for 2 hours. Paraffin-embedded retinal sections 7-μm thick were cut through the optic nerve and stained with hematoxylin and eosin. To quantify the number of neurons in the ganglion cell layer of the retina, cells were counted from one ora serrata through the optic nerve to the
other ora serrata. Optic nerves and lumbar spinal cords were postfixed in Zamboni’s fixative overnight. Paraffin-embedded sections that were 7-μm thick were stained with Luxol fast blue followed by hematoxylin and eosin staining.

Immunohistochemistry

Paraffin-embedded sections (7-μm thick) were examined using the following primary antibodies: mouse anti-NF200 (1:400; Sigma), mouse anti–glial fibrillary acidic protein (GFAP) (50 μg/mL; Progen, Heidelberg, Germany), goat anti-iba1 (1:400; Abcam, Cambridge, England), goat anti-TLR4 (2 μg/mL; sc-16240; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti–NF-κB p65 (1:500; catalog number 8242; Cell Signaling, Danvers, MA). The antibodies against TLR-4 and NF-κB have been widely used and described elsewhere. Stained sections were examined using a microscope (BX51; Olympus Corporation, Tokyo, Japan) connected to a DP70 camera (Olympus). Images were processed and viewed using DP manager software version 2.2.1.195 (Olympus). Quantitative analysis of the stained region was performed using ImageJ software version 1.46r (NIH, Bethesda, MD; http://imagej.nih.gov/ij).

Multifocal Electoretinogram Recordings

The mice were anesthetized by an i.p. injection of sodium pentobarbital (87.5 mg/kg). The pupils were dilated with 0.5% phenylephrine hydrochloride and 0.5% tropicamide. Multifocal electoretinograms were recorded using a VERIS 6.0 system (Electro-Diagnostic Imaging, Redwood City, CA). The visual stimulus consisted of seven hexagonal areas scaled with eccentricity. The stimulus array was displayed on a high-resolution black and white monitor, with a frame rate of 100 Hz. The second-order kernel of multifocal electoretinograms, which is a sensitive indicator of inner retinal dysfunction, was measured as previously reported.

Cell Culture

Primary astrocytes were obtained as previously reported. After two passages, the astrocytes were plated according to their intended purposes. The cells were pretreated with or without CV-11974 or SN50 (50 μg/mL) for 45 minutes, stimulated with Ang II for 6 or 24 hours, and subjected to quantitative PCR or immunoblot analysis. In the preliminary experiments, we used three concentrations of CV-11974 (100 nmol/L, 1 μmol/L, and 10 μmol/L) and found that 10 μmol/L of CV-11974 was the most effective. For Ang II, we tested two concentrations (10 nmol/L and 100 nmol/L) but found no marked difference between the two concentrations. Thus, we selected 10 μmol/L for CV-11974 and 10 nmol/L for Ang II for the whole study. To investigate the effects of Ang II on the production of monocyte chemotactic protein (MCP)-1 in astrocytes, the cells were pretreated with CV-11974 (10 μmol/L) and SN50 (50 μg/mL), followed by Ang II stimulation for 24 hours, and then stimulated with LPS (2 ng/mL) for 24 hours. The levels of secreted MCP-1 in the culture medium of astrocytes were determined by an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Real-Time Quantitative PCR

Real-time quantitative PCR was performed using an ABI 7500 fast real-time PCR system (Applied Biosystems,
Foster City, CA) with SYBR Green PCR Master Mix (Applied Biosystems), as previously reported. cDNA reverse transcribed from total RNA was amplified using primers specific for TLR2 (sense: 5'-CCCACTTCAAGGCTCTTGTAC-3'; antisense: 5'-GCCACTTCAAGGCTCTTGTAC-3'), TLR3 (sense: 5'-GTGCTACCGATCTCCTGGTT-3'; antisense: 5'-TTCCCAAGAGCTTCTGTT-3'), TLR4 (sense: 5'-GCCGAAAGGTATTGGTGAT-3'; antisense: 5'-TGCCATGTGGACCAATCTC-3'), TLR9 (sense: 5'-CAGCCCTGACTAGGGACAAC-3'; antisense: 5'-CGGAAACCAGCATGAAGAT-3'), glyceraldehyde-3-phosphate dehydrogenase (sense: 5'-GGATGCAGGGATGATGTTC-3'), and GAPDH (sense: 5'-GTGCATCGGATTCTTGGTTT-3').

To determine whether the RAS was involved in the pathogenesis of EAE, we first examined Ang II concentrations in mice with EAE. On the other hand, mice on day 10 after MOG immunization when the mice started to exhibit EAE signs (Figure 1C). These results suggest that the RAS has an important role during the development of EAE.

We then examined the effects of an orally active Ang II receptor antagonist candesartan on MOG-induced EAE in mice (Figure 1B). Histopathologic analysis of the optic nerves on day 25 revealed inflammatory cells and distinctive demyelination in optic nerve lesions in the vehicle-treated mice, whereas these findings were absent in the candesartan-treated mice with EAE (Figure 2A). These findings are consistent with those of previous reports. Furthermore, as revealed by NF200 staining, the rate of axonal survival was higher in the candesartan-treated mice with EAE, and the rate of GFAP-positive astrocytes and iba1-positive microglial cells were lower in this group (Figure 2B), indicating that candesartan administration ameliorated the severity of optic neuritis.

We next examined the neuroprotective effects of candesartan in the mice with EAE. In the vehicle-treated mice, the number of surviving neurons in the ganglion cell layer was significantly reduced, whereas candesartan treatment prevented cell loss (Figure 3, A and C). To determine whether the observed effects of candesartan on RGCs and optic nerves were functionally significant, we analyzed visual function using multifocal electoretinography, which is a vital tool for substantiating histologic observations of physiologic phenomena in the retina. The response topography revealed impaired visual function in the vehicle-treated mice with EAE in all visual fields, and visual function was partially improved by the candesartan treatment (Figure 3, B and C). These results suggested that the neuroprotective effects of candesartan in the mice with EAE were functionally significant.

As shown by the clinical scores, candesartan treatment significantly reduced the severity of EAE and delayed disease onset (Figure 4A), consistent with the findings of a previous report. However, it had no effects on the incidence of EAE (Figure 4B). Taken together, these data indicate that inhibition of Ang II exerts effects at histologic and functional levels, attenuating not only inflammation in spinal cords but also EAE-induced optic neuritis and RGC degeneration.

Candesartan Suppressed TLR4 Expression in Mice with EAE

We previously reported that Ang II increased TLR4 expression in retinal Müller glial cells in vitro and that candesartan treatment provided protection against RGC loss in a mouse model of normal tension glaucoma by inhibiting the TLR4 pathway. Previous studies found that upregulated expression of TLR4 was involved in EAE progression. To investigate whether Ang II increased TLR4 expression in astrocytes and whether candesartan suppressed this effect, we stimulated primary cultured astrocytes, which express AT1R and contribute to disease progression in MS and EAE, with Ang II. The real-time
quantitative PCR analysis revealed that Ang II up-regulated mRNA expression levels of TLR4 but not those of TLR2, TLR3, or TLR9. Furthermore, pretreatment with CV-11974, an in vitro antagonist of Ang II, completely abrogated this increase (Figure 5A). A Western blot analysis revealed similar findings (Figure 5B).

We next examined TLR4 mRNA expression in the spinal cord of mice with EAE and found that candesartan treatment suppressed EAE-induced up-regulation of TLR4 (Figure 6A). Moreover, double-labeling immunohistochemistry of TLR4 and GFAP revealed that TLR4 expression in astrocytes was significantly reduced in the candesartan-treated mice with EAE (Figure 6, B and C). Taken together, these data suggest that candesartan improves optic neuritis partially through the inhibition of innate immune responses in astrocytes.

**Ang II Increased TLR4 Expression via the NF-κB Pathway**

NF-κB is activated by TLR engagement and a variety of inflammatory signals. Because NF-κB can affect the incidence and severity of EAE, we investigated whether NF-κB was involved in Ang II–induced TLR4 expression. The results indicated that the Ang II treatment significantly increased nuclear translocation of NF-κB in astrocytes in vitro and that pretreatment with CV-11974 or SN50, an inhibitor of NF-κB nuclear translocation, suppressed the effects of Ang II (Figure 7A). Furthermore, preincubation with SN50 suppressed Ang II–induced up-regulation of TLR4 (Figure 7B). Double-labeling immunohistochemistry of NF-κB and GFAP in the spinal cord revealed that astrocytes with nuclear NF-κB expression were significantly reduced in the candesartan-treated mice with EAE (Figure 7, C and D).

**Figure 2** Candesartan suppressed demyelination, axonal degeneration, and neuroinflammation in optic nerves during experimental autoimmune encephalomyelitis (EAE). A: Optic nerves were stained with Luxol fast blue (LFB), hematoxylin and eosin (H&E), anti-NF200, anti–glial fibrillary acidic protein (GFAP), or anti-ib1 antibody. B: Quantitative analysis of demyelination, axonal degeneration, GFAP-positive areas, and ib1-positive areas in the optic nerves. Data are expressed means ± SEM, n = 7 (B). *P < 0.05, **P < 0.01. Scale bar = 110 μm (A).

**Figure 3** Candesartan ameliorated retinal ganglion cell loss and visual impairment induced by experimental autoimmune encephalomyelitis (EAE). A: Hematoxylin and eosin staining of representative retinal sections in healthy, vehicle, and candesartan-treated mice with EAE. B: Representative images of three-dimensional plots depicting averaged visual responses of the second-order kernel examined by a multifocal electroretinogram. The degree of retinal function is presented by the color bar. A higher score (red) indicates highly sensitive visual function, and a lower score (green) indicates retinal dysfunction. Values are given in nanovolts per square degree (nV/deg²). C: Quantitative analysis of cell numbers in the ganglion cell layer (GCL) (left panel) and visual response (right panel). Data are expressed as means ± SEM, n = 4 (C). *P < 0.05. Scale bars: 100 μm (A, top row); 70 μm (A, bottom row). INL, inner nuclear layer; ONL, outer nuclear layer.
We further investigated the functional significance of TLR4 up-regulation by Ang II in astrocytes. LPS-induced production of MCP-1 was significantly increased when astrocytes were pretreated with Ang II, but the addition of CV-11974 or SN50 suppressed this increase (Figure 7E), indicating that candesartan ameliorated the severity of EAE partially through the inhibition of chemokine expression in astrocytes.

Discussion

In the present study, we measured Ang II concentrations in serum and found that its concentration was significantly increased in the early phase of EAE in mice compared with that of healthy mice, pointing to an important role for the RAS during EAE. Circulating Ang II might act on immune cells, such as dendritic cells or T cells, to induce or increase the severity of EAE, whereas the Ang II receptor antagonist candesartan might attenuate EAE. To elucidate the role of the RAS in the CNS during EAE, further investigations of Ang II concentrations in cerebrospinal fluid, namely, concentrations of brain tissue—paracrine Ang II, are required.

We found that candesartan ameliorated inflammation-mediated degeneration in optic nerves and spinal cords. These findings are consistent with those of a previous report, which found that candesartan delayed the onset of MOG-induced EAE and attenuated disease severity. In addition, we found that Ang II up-regulated TLR4 expression in astrocytes via a NF-κB pathway and enhanced LPS-induced chemokine release from astrocytes and that candesartan suppressed the effects of Ang II. A previous study reported that the Ang II receptor antagonist inhibited up-regulation of molecules, such as TGF-β. TGF-β is a multipotent cytokine, and its role is determined by the surrounding milieu. In the CNS, TGF-β signaling is markedly up-regulated before and during the onset of EAE, creating a suitable environment for inflammation. Thus, inhibition of TGF-β up-regulation in the CNS by candesartan leads to attenuated severity in mice with EAE. In addition, candesartan may inhibit neuro-inflammation during optic neuritis by suppressing the activity of microglia, which have important roles in neuro-inflammation. We previously reported that inhibition of microglial activation ameliorated the severity of the effects of...
Rho-associated protein kinase via the NF-κB pathway. As reported previously, this pathway is particularly important in the migration of inflammatory cells, including microglia, to sites of inflammation. Additional studies are needed to elucidate details of the Ang II–NF-κB pathway.

**Figure 6** Candesartan suppressed up-regulation of Toll-like receptor (TLR)-4 in the spinal cords of mice with experimental autoimmune encephalomyelitis (EAE). **A:** Quantitative PCR analysis of TLR4 in the spinal cords of mice with EAE with or without candesartan treatment. **B:** Quantitative analysis of TLR4 and glial fibrillary acidic protein (GFAP) double-positive areas in C. **C:** Immunohistochemistry using antibodies for TLR4 (red) and GFAP (green) in the spinal cord of mice with EAE with or without candesartan treatment. Data are expressed as means ± SEM. *n* = 4 (A and B). *P* < 0.05, **P* < 0.01, and ***P* < 0.001. Scale bar = 40 μm.

EAE on the spinal cord and optic nerves. Research also found that Ang II enhanced TLR4-mediated signaling in microglia, induced microglial activation, and increased the production of reactive oxygen species within the paraventricular nucleus. Moreover, Ang II was found to play a major role in microglia polarization toward a proinflammatory and classically activated (M1) phenotype. In the present study, we found another function of candesartan: inhibition of enhanced chemokine release from astrocytes. This function might partially explain its neuroprotective effects during optic neuritis. Our findings, together with those in the literature, suggest that candesartan inhibits inflammation in the CNS through multiple pathways during EAE. It would be interesting to determine whether the incidence in MS in people routinely taking ACE inhibitors or Ang II receptor antagonists is low.

NF-κB is a ubiquitous transcription factor that plays an important role in controlling the expression of genes involved in immunity, inflammation, cell proliferation, and cell apoptosis. In the present study, we found that TLR4 expression was up-regulated by the Ang II–NF-κB pathway (Figure 7). Interestingly, a previous study reported that Ang II induced an increase in the activity of NF-κB–Toll-like receptor (TLR)-4 signaling. A: Immunoblot analysis of NF-κB in nuclear fractions of astrocytes treated with CV-11974 (CV) or SN50 for 45 minutes followed by stimulation with angiotensin II (Ang II) for 1 hour. **B:** Immunoblot analysis of TLR4 in astrocytes treated with SN50 for 45 minutes followed by Ang II stimulation for 24 hours. **C:** Immunohistochemistry using antibodies for NF-κB (red) and glial fibrillary acidic protein (GFAP) (green) in the spinal cord of mice with EAE with or without candesartan treatment. **D:** Quantitative analysis of NF-κB and GFAP double-positive cells in panel C. **E:** Effects of CV and SN50 on the production of monocyte chemotactic protein (MCP)-1 in astrocytes pretreated with Ang II for 24 hours followed by lipopolysaccharide (LPS) stimulation for 24 hours. Data are expressed as means ± SEM for a representative experiment run in quadruplicate (A, B and E); means ± SEM (D). *n* = 4 (D). *P* < 0.05, **P* < 0.01, and ***P* < 0.001. Scale bar = 40 μm (C).
However, this result of TLR4 up-regulation by the Ang II–NF-κB pathway seems to be interesting because NF-κB is also activated by TLR pathways and a variety of inflammatory signals, such as interleukin-1β and tumor necrosis factor-α. These findings suggest that NF-κB is involved in the pathogenesis of MS and EAE upstream and downstream of TLR signaling.

Other downstream molecules of TLR signaling pathways, such as myeloid differentiation factor 88 and apoptosis signal-regulating kinase (ASK)-1, are also important during such as myeloid differentiation factor 88 and apoptosis downstream of TLR signaling.

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