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The Renin-Angiotensin System Regulates Neurodegeneration in a Mouse Model of Optic Neuritis

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The major role of the renin-angiotensin system (RAS), including that of angiotensin II (Ang II), the principal effector molecule, in the cardiovascular system is well known. Increasing evidence suggests that the RAS also plays a role in the development of autoimmune diseases. Optic neuritis (ie, inflammation of the optic nerve, with retinal ganglion cell loss) is strongly associated with multiple sclerosis. We investigated the effects of candesartan, an Ang II receptor antagonist, on optic neuritis in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. The Ang II concentration was increased in the early phase of EAE. Oral administration of candesartan markedly attenuated demyelination of the optic nerve and spinal cord and reduced retinal ganglion cell loss and visual impairment in mice with EAE. *In vitro* analyses revealed that Ang II up-regulated the expression of Toll-like receptor (TLR)-4 in astrocytes via the NF-κB pathway. In addition, Ang II treatment enhanced lipopolysaccharide-induced production of monocyte chemoattractant protein 1 in astrocytes, and pretreatment with candesartan or SN50, an NF-κB inhibitor, suppressed the effects of Ang II. The novel pathway of RAS—NF-κB—TLR4 in glial cells identified in the present study may be a valid therapeutic target for neurodegeneration in neuroinflammatory diseases. *(Am J Pathol 2017, ■: 1—10; https://doi.org/10.1016/j.ajpath.2017.08.012)*

Q3 Optic neuritis refers to inflammation of the optic nerve, with retinal ganglion cell (RGC) loss.[1–3] An epidemiologic study in the United States estimated that the annual incidence rate of optic neuritis was 5 per 100,000 population.[4] 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.[1] Optic neuritis can result in severe, irreversible visual loss, especially the optic-spinal form of MS or neuromyelitis optica.[5] 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.[6] 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.[6] 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).[7] Currently, Ang II receptor antagonists and ACE inhibitors are used as prescribed drugs to treat high blood pressure.[8] In addition, recent studies implicated the RAS in the development of autoimmune diseases.[9–12] 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 MS, inhibition of these components produced in different steps of the RAS system all markedly ameliorated the course of EAE.[9–11] Moreover, Ang II has been identified as a

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paracrine mediator, which sustains inflammation in the CNS by up-regulating transforming growth factor (TGF)-β.[13] AT1Rs were reported to be primarily expressed in CNS-resident cells during EAE.[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.[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.[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.[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.[17,18] Consistent with these reports, *in vivo* treatment with specific TLR ligands exacerbated EAE, whereas targeted disruption of selected TLRs prevented EAE.[17,19–21] These results suggest that TLRs have critical roles in MS and EAE.[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.[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.[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.[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.[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. [F1] EAE was induced in mice using the myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK), as previously reported.[26] Briefly, the mice received s.c. injections with 100 μg of $MOG_{35–55}$ 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.[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

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