

# Neuroprotective Effects of Angiotensin II Type 1 Receptor (AT1R) Blocker, Telmisartan, via Modulating AT1R and AT2R Signaling in Retinal Inflammation

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**PURPOSE.** To investigate the retinal neural damage that occurs during inflammation and the therapeutic effects of the angiotensin II type 1 receptor (AT1R) blocker, telmisartan, using a model of endotoxin-induced uveitis (EIU).

**METHODS.** The localization of AT1R and AT2R was shown by immunohistochemistry. EIU was induced by intraperitoneal injection of lipopolysaccharide (LPS). Animals were treated with telmisartan for 2 days and were evaluated 24 hours later. Expression levels of angiotensin II, STAT3 activation induced by inflammatory cytokines, and retinal proteins essential for neural activities (e.g., synaptophysin, rhodopsin) were analyzed by immunoblot. An AT2R antagonist was administered to evaluate the contribution of AT2R signaling in this therapy. Dark-adapted full-field electroretinography (ERG) was also performed.

**RESULTS.** AT1R and AT2R were expressed in presynaptic terminals in most of the retinal neurons. AT1R was also expressed in Müller glial cells. During inflammation, angiotensin II expression was elevated, STAT3 was activated, and synaptophysin and rhodopsin expression were reduced. The expression of glial fibrillary acidic protein (GFAP), downstream of STAT3 activation, was induced in Müller glial cells. However, treatment with telmisartan successfully avoided all these changes. An AT2R antagonist lowered synaptophysin expression despite the treatment. STAT3 activity was negatively correlated with rhodopsin expression. Furthermore, ERG responses, which were mostly prevented by telmisartan, were disturbed during inflammation.

**CONCLUSIONS.** Retinal protein expression and visual function are both disturbed by inflammation. Treatment with the AT1R blocker telmisartan efficiently prevented these signs of retinal neural damage through the reduction of local angiotensin II expression, the blockade of AT1R, and the relative upregulation

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Inflammatory reactions are involved in most retinal diseases, among them diabetic retinopathy and vascular occlusive diseases, leading to visual loss.<sup>1,2</sup> During inflammation, various kinds of cytokines, such as interleukin (IL)-6<sup>3-5</sup> and tumor necrosis factor (TNF)- $\alpha$ ,<sup>3</sup> have been reported to cause pathologic changes. In addition to these cytokines, angiotensin II,<sup>2,6-10</sup> conventionally known as a regulator of salt and water retention and of systemic blood pressure, has received attention as a modulator of inflammation.<sup>2,7,11</sup> It is produced in many organs and in ocular tissue<sup>8,9</sup> from angiotensinogen through the renin/angiotensin system (RAS). Angiotensin II is highly expressed intraocularly in human diabetic retinopathy,<sup>8-10,12</sup> suggesting that it is involved in the retinal disease process.

We have already reported that angiotensin II causes pathologic changes in the retinal vascular system through the angiotensin II type 1 receptor (AT1R), which mainly mediates the angiotensin II signal.<sup>2,13</sup> On the other hand, AT1R signaling also affects neural synaptic activity in the brain.<sup>14-17</sup> Thus, angiotensin II may damage retinal neural cells during inflammation, but the exact influences of this signal and the effects of the AT1R blocker on retinal neural cells remain to be elucidated. Another angiotensin II receptor, AT2R, acts in a manner opposite that of AT1R signaling, especially under stress conditions, and possibly plays a role<sup>13</sup> in this therapy, but it also remains obscured.

In this study, we first described that AT1R and AT2R are expressed in most retinal neural cells, including synapses. Then we evaluated the influences of inflammation with excessive angiotensin II and the effects of treatment with telmisartan on retinal neural cells using animal model of lipopolysaccharide (LPS)-induced inflammation. This is also known as a model of endotoxin-induced uveitis (EIU) in which pan-retinal vasculitis occurs<sup>2</sup> and various kinds of cytokines, such as IL-6,<sup>2,18</sup> are induced. In addition, angiotensin II is upregulated in the retina, as we show in this study.

To evaluate retinal damage, we analyzed the retinal neural proteins synaptophysin and rhodopsin, which are essential for visual function. Synaptophysin is a presynaptic vesicle protein that controls neurotransmitter release. Rhodopsin is the major visual substance we have recently found to be negatively regulated by STAT3 activation in the neonatal retina.<sup>19,20</sup> Although AT1R is well known to be coupled with G-protein, it is also in the upstream of the JAK/STAT pathway.<sup>21,22</sup> Thus, we analyzed whether a similar response occurs in the adult retina. Given that excessive angiotensin II expressed during inflammation may selectively bind to AT2R after AT1R blockade, the contribution of AT2R signaling in this therapy is also evaluated by the administration of an AT2R-specific antagonist. Finally, we perform electroretinography (ERG), a common and objective clinical method for estimating visual function.

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All our data show that AT1R, expressed in most of the retinal neural cells, plays key roles in retinal neural damage during inflammation. We have already reported the possibility of applying the AT1R blocker, telmisartan, in treating retinal vascular inflammation, and the present study demonstrates that it is also effective in protecting physical activities of the neural retina during inflammation.

## MATERIALS AND METHODS

### Animals

C57BL/6 mice (8 weeks old) were purchased (Clea Japan, Tokyo, Japan). All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Each mouse received a single intraperitoneal injection of 6.0 mg/kg body weight of lipopolysaccharide (LPS) from *Escherichia coli* (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS). Mice were killed and evaluated 24 hours after LPS injection. This time point was chosen for analysis because most of the pathologic changes in the retina were obvious by this time. Mice were treated with intraperitoneal injections of vehicle (0.25% dimethyl sulfoxide [DMSO] in PBS) or the AT1R blocker, telmisartan (120  $\mu$ M in 0.25% DMSO, 20 mg/kg body weight; a gift of Boehringer Ingelheim, Ingelheim, Germany) on the day before and the day of the LPS injection. The AT2R blocker, PD123319<sup>23</sup> (10 mg/kg body weight; Tocris Cookson, Ltd., Bristol, UK) and the peroxisome proliferation-activated receptor  $\gamma$  (PPAR- $\gamma$ ) antagonist GW9662<sup>24</sup> (10 mg/kg body weight; Alexis Biochemicals, San Diego, CA) were intraperitoneally injected when telmisartan was injected.

### Immunohistochemistry

Cryosections (14–16  $\mu$ m) were prepared by perfusing mice with 4% paraformaldehyde (PFA), as described previously.<sup>19</sup> For immunostaining of rabbit anti-AT1R (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-AT2R (1:100, Santa Cruz Biotechnology), and mouse anti-synaptophysin (1:100 Sigma), sections were incubated with 1% H<sub>2</sub>O<sub>2</sub> in PBS for 20 minutes and at 4°C overnight with the primary antibody diluted in blocking reagent with 0.1% Triton. The sections were then incubated with biotin-conjugated goat anti-rabbit IgG (1:500; Chemicon International, Temecula, CA) or biotin-conjugated goat anti-mouse IgG (1:500; Vector Laboratories, Burlingame, CA) and with a prepared avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit; Vector Laboratories) and then were detected with a tyramide signaling amplification (TSA) fluorescein system (PerkinElmer Life Science, Boston, MA). For immunostaining of rabbit anti-glial fibrillary acidic protein (anti-GFAP, 1:1000; DAKO, Carpinteria, CA) and mouse anti-neurofilament (1:100, Roche), mouse anti-glutamine synthetase (1:400 Molecular Probes, Eugene, OR), sections were incubated and detected with Alexa 568-conjugated goat anti-rabbit, or Alexa 488-conjugated goat anti-mouse IgG (1:500; Molecular Probes), respectively. Nuclei were stained with the nuclear dye bisbenzimidazole 1:1000 from a stock solution of 10 mg/mL stain (Hoechst 33258; Sigma). Sections were examined with a laser scanning confocal microscope (LSM510; Carl Zeiss, Jena, Germany).

### Immunoblot Analysis

Mice were killed with an overdose of anesthesia. The eyes were immediately enucleated, and the retina was carefully isolated and placed in lysis buffer. The lysate was separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Bedford, MA). After blocking with 4% skim milk, the membranes were incubated overnight with rabbit anti-angiotensin II antibody (1:200; Peninsula Laboratories, Belmont, CA), rabbit anti-AT1R antibody (1:100; Santa Cruz Biotechnology), mouse anti-synaptophysin antibody (1:500; Sigma), rabbit anti-phosphorylated-STAT3

antibody (1:1000; Cell Signaling Technology, Beverly MA), rabbit anti-rhodopsin antibody (1:10,000; Cosmo Bio, Tokyo, Japan), and anti- $\alpha$ -tubulin (1:2000; Sigma) to equalize the amount of protein in each sample, respectively. Membranes were then incubated with biotin-conjugated secondary antibodies followed by avidin-biotin complex (Vectastain ABC Elite Kit; Vector Laboratories) or horseradish peroxidase-conjugated secondary antibodies. Finally, they were detected through enhanced chemiluminescence (ECL Blotting Analysis System; Amersham, Arlington Heights, IL) and measured by a NIH image. Statistical analysis was performed by the Bonferroni/Dunn test.

### ERG

Mice were dark adapted for at least 12 hours and prepared under dim red illumination, anesthetized with 70 mg/kg body weight of pentobarbital sodium (Dainippon Sumitomo Pharmaceutical Co., Osaka, Japan), and placed on a heating pad throughout the experiment. The pupils were dilated with one drop of a mixture of 0.5% tropicamide and 0.5% of phenylephrine (Santen Pharmaceutical Co., Osaka, Japan). The ground electrode was a needle placed subcutaneously in the tail, and the reference electrode was placed subcutaneously between the eyes. The active electrodes were gold wires placed on the cornea. Recordings were performed (PowerLab System 2/25; AD Instruments, New South Wales, Australia). Responses were differentially amplified and filtered through a digital bandpass filter ranging from 0.313 to 1000 Hz to yield a- and b-waves. Light pulses of 800 cd  $\cdot$  s/m<sup>2</sup> and 4-ms duration were delivered through a commercial stimulator (Ganzfeld System SG-2002; LKC Technologies, Inc., Gaithersburg, MD). Electrode impedance was checked before and after each measurement in all animals with the use of the machine's built-in feature. The implicit time of the a- and b-waves was measured from the onset of stimuli to the peak of each wave. Three researchers measured the amplitude of the a-wave from baseline to the trough of the a-wave and the amplitude of the b-wave from the trough of the a-wave to the peak of the b-wave. Statistical analysis was carried out with the Fisher PLSD test.

## RESULTS

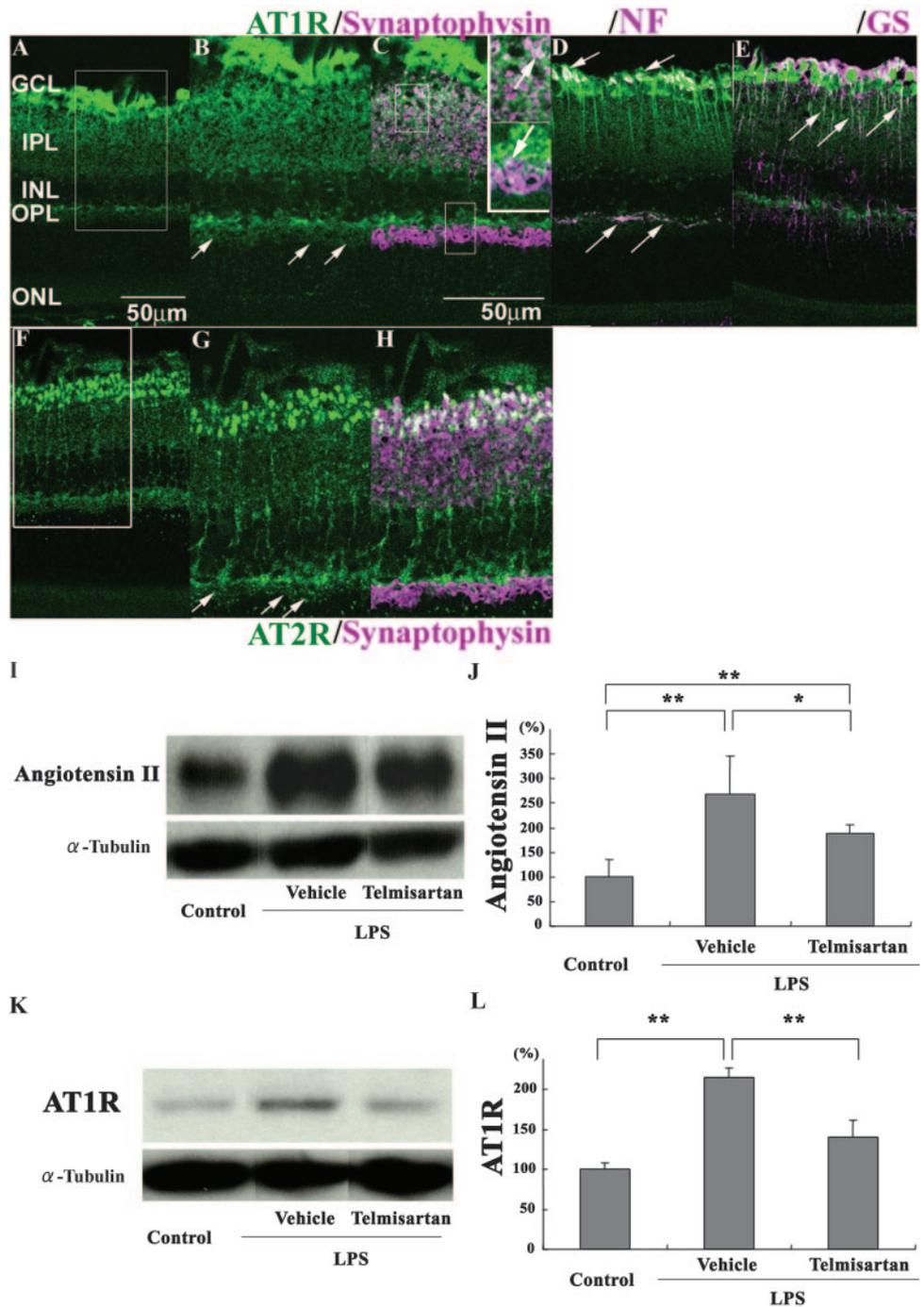
### Expression of AT1R and AT2R in the Retina

We first analyzed the expression of AT1R and AT2R in the retina of adult mice under physiological conditions. AT1R expression was observed in the inner plexiform layer (IPL), outer plexiform layer (OPL), and retinal ganglion cell layer (GCL; Fig. 1A). AT1R in the IPL and OPL was coexpressed with synaptophysin, a presynaptic vesicle membrane protein (Figs. 1B, 1C, insets), indicating that AT1R is expressed in synapses. This is consistent with previous reports that AT1R is expressed in the presynaptic terminals of the neurons, where it modulates the kinetics of the synaptic vesicles.<sup>14–17,25</sup> It includes synapses in the rod photoreceptor cells (B, arrows) judging from the localization. AT1R was also coexpressed with neurofilament (Fig. 1D) and was thus expressed in nerve fibers in the GCL and OPL. Moreover, it was coexpressed with glutamine synthetase (Fig. 1E), indicating that AT1R is also expressed in Müller glial cells. Some of the AT1R expression in the GCL seemed to be present in vascular endothelial cells, judging from its structure, as reported previously.<sup>2</sup>

AT2R, which is known to function in response to stress, was clearly expressed in the IPL and OPL under control conditions (Fig. 1F). Most of the AT2R was coexpressed with synaptophysin, indicating that AT2R is present in synaptic sites (Figs. 1G, 1H). The most intense staining for AT2R in the innermost IPL was double positive with anti-PKC $\alpha$ , one of the rod bipolar cell markers (data not shown). AT2R expression was also slightly observed in nerve fibers in the GCL.

Next we compared the expression levels of angiotensin II, AT1R, and AT2R in the retina during inflammation with or without treatment using telmisartan. Expression of angiotensin

**FIGURE 1.** Expression of AT1R and AT2R in the retinas of adult mice under control conditions. AT1R (A) was coexpressed with synaptophysin, a presynaptic vesicle protein, in the IPL and OPL (marked area in (A) was magnified in (B) and (C) and merged in (C)). Coexpression of AT1R (green) and synaptophysin (red) was clearly observed in the magnified images of the IPL and OPL (C, arrows in insets). AT1R was probably expressed in photoreceptor cells judging from the localization (B, arrows). AT1R was also coexpressed with neurofilament, a marker for nerve fibers in the GCL and OPL (D, arrows), and glutamine synthetase, a marker for Müller glial cells (E, arrows). AT2R was mainly expressed in the IPL and OPL (F), most of which were coexpressed with synaptophysin (marked area in (F) was magnified in (G) and (H) and merged in (H)). AT2R was probably expressed in photoreceptor cells (G, arrows). It was slightly observed in nerve fibers in the GCL (F). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; NF, neurofilament; GS, glutamine synthetase. Immunoblot analyses. Expression of angiotensin II (I, J) was upregulated by LPS injection and suppressed after treatment with telmisartan, though it remained at a level higher than control (Control: LPS/LPS + telmisartan; 1.00:2.68:1.88;  $n = 6:6:8$ ;  $*P < 0.05$ ;  $**P < 0.01$ ; Bonferroni/Dunn test (I, J)). Expression level of AT1R (Control: LPS/LPS + telmisartan; 1.00:2.15:1.41;  $n = 6:6:8$ ;  $**P < 0.05$ ; Bonferroni/Dunn test (K, L)) was upregulated in LPS-induced inflammatory retina, whereas it was downregulated after treatment with telmisartan.

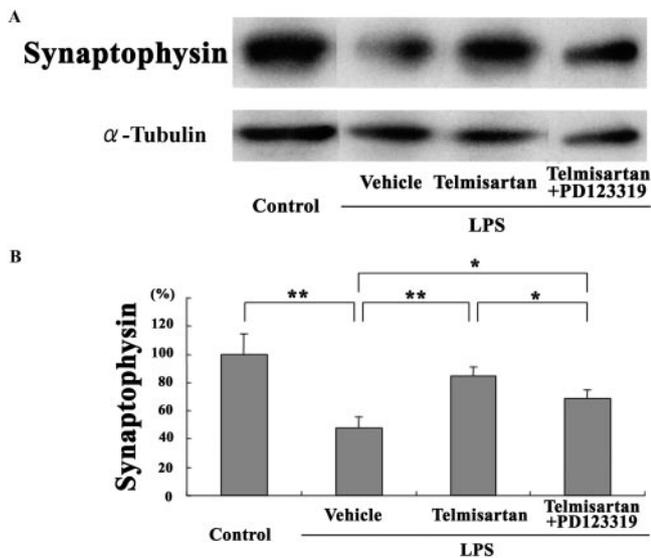


II was 2.7-fold upregulated by LPS injection, which was significantly suppressed by treatment with telmisartan, although it remained upregulated compared with control (control, LPS/LPS + telmisartan; 1.00:2.68:1.88; Figs. 1I, 1J).

AT1R expression was also upregulated during inflammation and was clearly suppressed by treatment with telmisartan (control, LPS/LPS + telmisartan; 1.00:2.15:1.41; Figs. 1K, 1L). This was consistent with the report in the brain<sup>26,27</sup> that AT1R expression is upregulated by a positive feedback system. Thus, the administration of AT1R blocker efficiently cut off the feedback loop. On the other hand, AT2R expression was 2.8-fold upregulated after LPS injection and was still maintained at a high level (2.3-fold) after treatment with telmisartan (data not shown).

### Protective Effect of Telmisartan on Synaptophysin Expression during Inflammation

Both AT1R and AT2R were well expressed in synapses in the retina. To investigate the effect of angiotensin II on synapses, we analyzed the expression level of synaptophysin. The expression of synaptophysin during LPS-induced inflammation was significantly reduced, but it was clearly prevented by telmisartan. To evaluate the possible contribution of AT2R signaling in telmisartan treatment, we next injected an AT2R antagonist, PD123319, in addition to LPS and telmisartan. The expression of synaptophysin was lower after PD123319 injection even after LPS-induced inflammation was treated with telmisartan, though the level was clearly higher compared with



**FIGURE 2.** Reduction in synaptophysin expression during inflammation was prevented by telmisartan. Synaptophysin expression during retinal inflammation was significantly reduced, but telmisartan clearly prevented the change. Restored synaptophysin expression by telmisartan was partially cancelled after administration of the AT<sub>2</sub>R antagonist PD123319 (Control: LPS/LPS + telmisartan/LPS + telmisartan + PD123319; 1:0.48:0.85:0.69;  $n = 6:5:5:5$ ;  $**P < 0.01$ ;  $*P < 0.05$ ; Bonferroni/Dunn test (A, B)).

the LPS-induced inflammatory retina with no treatment (control, LPS/LPS + telmisartan/LPS + telmisartan + PD123319; 1:0.48:0.85:0.69;  $n = 6:5:5:5$ ;  $**P < 0.01$ ;  $*P < 0.05$ ; Bonferroni/Dunn test; Figs. 2A, 2B). This suggested that the upregulation of AT<sub>2</sub>R function relative to AT<sub>1</sub>R was involved in rescuing the expression of synaptophysin in this therapy.

Given that additional partial PPAR- $\gamma$  agonist activity is spontaneously found in telmisartan,<sup>28,29</sup> its contribution was also analyzed by administration of the PPAR- $\gamma$  antagonist, GW9662, in addition to telmisartan. Although a high dose of GW9662 was injected compared with previous reports,<sup>24,30,31</sup> the level of synaptophysin expression was not changed (data not shown), suggesting that there was less contribution of PPAR- $\gamma$  activity in this case.

Thus, the AT<sub>1</sub>R blocker telmisartan inhibited the reduction of synaptophysin expression during retinal inflammation, by the blockade of AT<sub>1</sub>R and the relative upregulation of AT<sub>2</sub>R function.

### Protective Effect of Telmisartan on Rhodopsin Expression during Inflammation with Reduction of Activated STAT3

After LPS injection, the expression of angiotensin II was upregulated (Figs. 1I, 1J), and in its downstream, the inflammatory cytokine IL-6<sup>2,32</sup> was induced, as we have previously reported.<sup>2</sup> Both ligands can activate STAT3,<sup>33</sup> which causes multiple events, depending on the cell type. Immunoblot analysis showed that STAT3 was highly activated in the neural retina but that it was significantly prevented by telmisartan (control, LPS/LPS + telmisartan; 1.00:1.92:1.51; Figs. 3A, 3B). Although AT<sub>2</sub>R<sup>21</sup> and PPAR- $\gamma$ <sup>34</sup> activity has been reported to inhibit STAT3 activation biochemically, neither receptor activity showed any contribution (data not shown). Therefore, strong STAT3 activation was induced by retinal inflammation but was prevented by AT<sub>1</sub>R blockade.

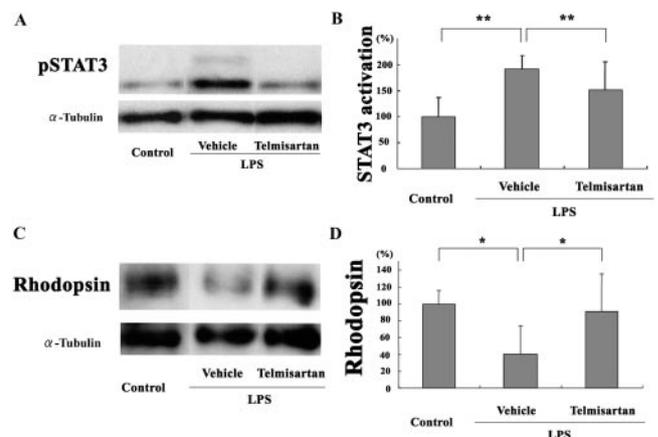
Next, we analyzed whether rhodopsin expression negatively correlated with STAT3 activation, as it did in the neonatal

retina. During inflammation with intense STAT3 activation, rhodopsin expression was significantly downregulated toward 40% of the control condition. However, treatment with telmisartan, which avoided excessive STAT3 activation, successfully preserved rhodopsin expression (control, LPS/LPS + telmisartan; 1.0:0.40:0.91; Figs. 3C, 3D). Because immunohistochemical findings showed that the number of photoreceptor cells was not altered and that TUNEL-positive cells were rarely observed in this model (data not shown), each rod photoreceptor cell might have reduced the level of rhodopsin expression. The contribution of AT<sub>2</sub>R function or PPAR- $\gamma$  activity was not observed (data not shown). Thus, rhodopsin expression was attenuated during inflammation and negatively correlated with strong STAT3 activation, which was efficiently prevented by AT<sub>1</sub>R blockade.

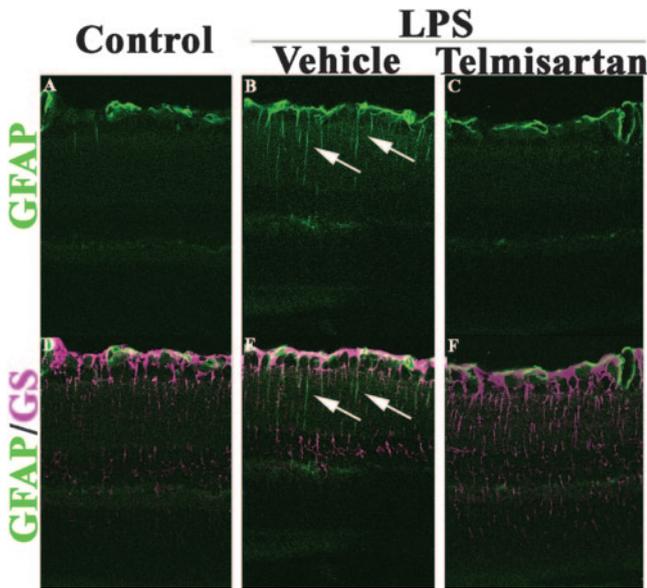
In the retina, Müller glial cells maintain the microenvironment, but, when pathologic events occur, they alter their characteristics to become reactive glial cells. It is recognized by GFAP upregulation,<sup>35</sup> which is induced by STAT3 activation.<sup>35–37</sup> Under control conditions, GFAP expression (Fig. 4A) in Müller glial cells (Fig. 4D) was only observed in their endfeet but was clearly induced through the columnar cell bodies when inflammation occurred (Figs. 4B, 4E). However, this was mostly avoided by treatment with telmisartan (Figs. 4C, 4F). These results suggested that STAT3 was also activated in Müller glial cells through the AT<sub>1</sub>R pathway.

### Protective Effect of Telmisartan on Visual Responses after Retinal Inflammation

We next recorded full-field ERG after 12 hours of dark adaptation (Figs. 5A–E). Overall responses were reduced after inflammation but preserved by treatment with telmisartan. Amplitude of the a-wave was significantly lowered (control, LPS/LPS + telmisartan; 311:159:282 ( $\mu$ V); Figs. 5A, 5B), and the implicit time of the b-wave was prolonged (control, LPS/LPS + telmisartan; 44.7:83.5:65.3 (ms); Figs. 5A, 5E) after inflammation, but those changes were not observed after treatment with telmisartan. Therefore, functional analysis also showed that visual function was successfully rescued from retinal damage during inflammation by the AT<sub>1</sub>R blocker telmisartan.



**FIGURE 3.** Induction of STAT3 activation and reduction of rhodopsin expression during inflammation were effectively blocked by telmisartan. STAT3 activation induced during inflammation was inhibited by telmisartan (Control: LPS/LPS + telmisartan; 1.00:1.92:1.51;  $n = 6:6:7$ ;  $**P < 0.01$ ; Bonferroni/Dunn test (A, B)). Rhodopsin expression was significantly suppressed during inflammation, which was clearly avoided by treatment with telmisartan (Control: LPS/LPS + telmisartan; 1.00:0.40:0.906;  $n = 6:7:9$ ;  $*P < 0.05$ ; Bonferroni/Dunn test (C, D)).



**FIGURE 4.** GFAP expression during inflammation was suppressed by telmisartan. GFAP expression (A–C) in Müller glial cells (D–F, detected by GS) was only observed in their endfeet in control conditions (A, D), but it was clearly upregulated through the columnar cell bodies during inflammation (B, E), which was observed less often after treatment with telmisartan (C, F). GFAP, glial fibrillary acidic protein; GS, glutamine synthetase.

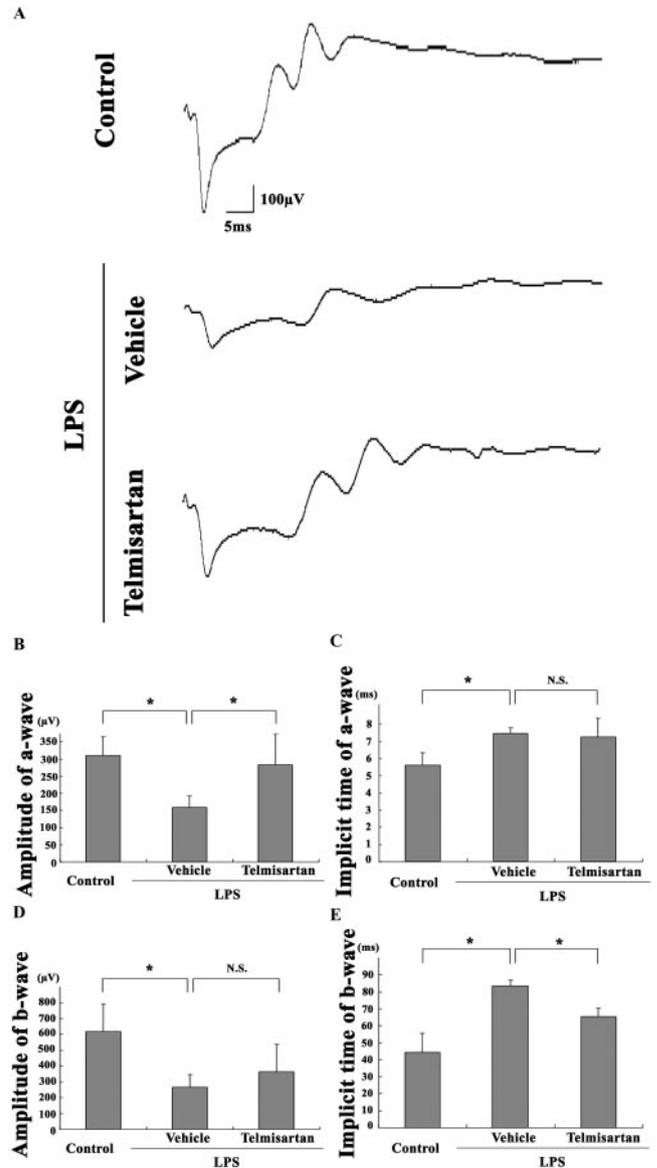
## DISCUSSION

We demonstrated that intense inflammation caused local upregulation of angiotensin II expression and thereby reduced the expression levels of rhodopsin and synaptophysin. We also showed severe disturbance of visual function by ERG. Treatment with the AT1R blocker telmisartan effectively prevented pathologic changes through the reduction of local angiotensin II expression, blockade of AT1R signaling with or without subsequent activation of STAT3, and relative upregulation of AT2R function. Both AT1R and AT2R are expressed in most of the retinal neural cells (Fig. 1), suggesting the possibility that telmisartan directly affects retinal neural cells.

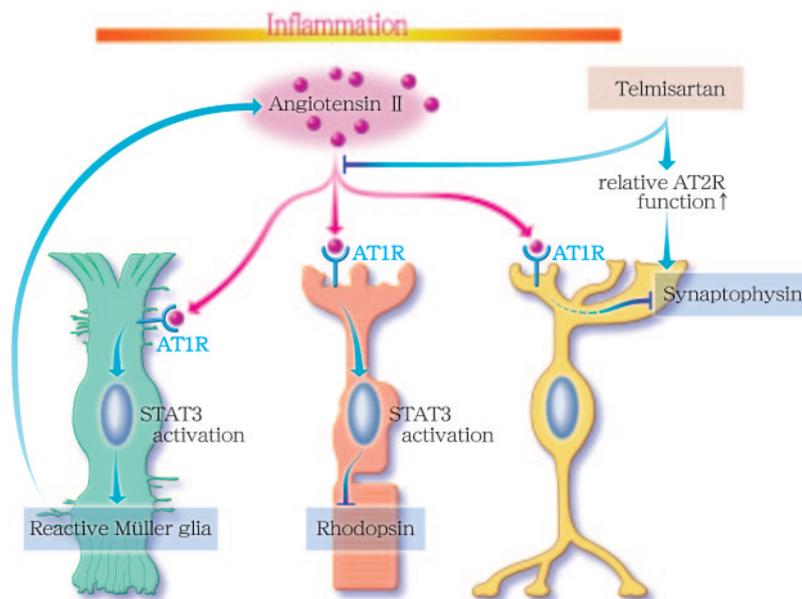
High levels of angiotensin II were observed in the retina of this model with retinal inflammation. It is generally agreed that continuous activation of local RAS causes tissue inflammation rather than circulating angiotensin II.<sup>9,38,39</sup> This is also the case in the retina, and all the components for RAS are inducible in the neural retina or surrounding tissues.<sup>8,9,10,12,40,41</sup> In particular, the first substrate of RAS, angiotensinogen, is expressed in Müller glial cells<sup>9,40</sup> and is upregulated by STAT3 activation.<sup>42,43</sup> As shown in this study, STAT3 should be activated in Müller glial cells at least through AT1R during inflammation; thus, a high level of angiotensinogen may be induced during retinal inflammation. In addition, the expression of AT1R itself was also increased so that once triggered, angiotensin II could be produced continuously through AT1R in the retina. Thus, local angiotensin II expression in retinal tissue was efficiently inhibited by AT1R blockade in Müller glial cells. Therefore, one of the therapeutic targets for the AT1R blocker during retinal inflammation included reduction in local angiotensin II expression by cutoff of the positive feedback loop of locally activated RAS (Fig. 6).

Several changes were observed in retinal neurons and Müller glial cells under high levels of angiotensin II during inflammation that also appeared to be good therapeutic targets for the AT1R blocker telmisartan (Fig. 6).

The most obvious effect during inflammation was the reduction of rhodopsin expression, which should have disturbed the function of rod photoreceptor cells but was mostly prevented by treatment with the AT1R blocker telmisartan. The level of rhodopsin expression was negatively correlated with STAT3 activation, as in the neonatal retina.<sup>19,20</sup> Although rod photoreceptor cells express several kinds of cytokine receptors that can activate STAT3, STAT3 activation in photoreceptor cells is rarely observed under normal conditions.<sup>19,20</sup> In the



**FIGURE 5.** Abnormalities in ERG response during inflammation were markedly prevented by telmisartan. Full-field electroretinography (ERG) after 12 hours of dark adaptation (A). Overall responses were reduced in LPS-induced inflammatory retina but were mostly preserved after treatment with telmisartan. Amplitude and implicit time of a- and b-waves were significantly altered during inflammation and, notably, were saved by treatment. Amplitude of a-wave; control: LPS/LPS + telmisartan; 311:159:282 ( $\mu\text{V}$ );  $n = 4:3:4$ ;  $*P < 0.05$ , Fisher PLSD test (B). Implicit time of a-wave; control: LPS/LPS + telmisartan; 5.63:7.45:7.23 (ms);  $n = 4:3:4$ ;  $*P < 0.05$ , Fisher PLSD test (C). Amplitude of b-wave; control: LPS/LPS + telmisartan; 616:267:361 ( $\mu\text{V}$ );  $n = 4:3:4$ ;  $*P < 0.05$ , Fisher PLSD test (D). Implicit time of b-wave; control: LPS/LPS + telmisartan; 44.7:83.5:65.3 (ms);  $n = 4:3:4$ ;  $*P < 0.05$ , Fisher PLSD test (E).



**FIGURE 6.** Model of mechanisms affecting retinal neural activities by angiotensin II during inflammation and therapeutic targets of telmisartan. Angiotensin II is highly upregulated during inflammation and dominantly binds to AT1R in the retinal neural cells. Through AT1R, STAT3 activation is induced in the Müller glial cells, upregulating GFAP expression and further increasing angiotensin II production. Thus, this inflammatory reaction is accelerated in a positive feedback manner. AT1R signaling also induces STAT3 activation in the rod photoreceptor cells, reducing rhodopsin expression. AT1R signaling downregulates synaptophysin expression in the retinal neurons. AT1R blockade with telmisartan successfully prevents these signs of retinal neural damage. In addition, the relative upregulation of AT2R function after AT1R blockade also contributes to the preservation of synaptophysin expression.

present study, however, the excessive stimuli should have activated STAT3 in the photoreceptor cells under pathologic conditions, as follows: AT1R signaling induces IL-6 expression in the retina<sup>2</sup> through NF- $\kappa$ B, and, in turn, IL-6 upregulates AT1R expression.<sup>44</sup> Both angiotensin II and IL-6 can activate STAT3; thus, excessive ligands should have forced STAT3 to activate synergistically in rod photoreceptor cells, which disturbs rhodopsin expression in adults. Conversely, the AT1R blocker suppressed the expression of these ligands and directly blocked AT1R, thus avoiding STAT3 activation in rod photoreceptor cells and reduction in rhodopsin expression.

Furthermore, synaptophysin expression during inflammation was reduced, which should have affected synaptic function by disturbing the exo-endocytosis of the synaptic vesicle essential for releasing neurotransmitters.<sup>45</sup> However, it was also averted by the AT1R blocker, telmisartan. Although AT2R was already expressed in synapses and has a level of affinity to angiotensin II almost identical with that of AT1R,<sup>13</sup> AT2R signaling could not eliminate the pathologic changes caused by AT1R signaling without treatment with the AT1R blocker, possibly because the expression level of AT2R was basically lower than that of AT1R. However, AT1R blockade probably led to the selective binding of angiotensin II to AT2R instead of AT1R. Moreover, the expression level of AT2R was not downregulated by AT1R blocker, which was advantageous for this therapy.

The mechanisms to rescue synaptophysin expression may be through direct AT1R signaling because AT1R signaling has been reported to control the kinetics of synaptic vesicles<sup>25</sup> and the expression of their components,<sup>15</sup> which can be cancelled by AT2R signaling.<sup>46</sup> Excessive AT1R signaling may promote the exocytosis of synaptic vesicles beyond the capacity of the re-uptake system to exhaust synaptophysin or to suppress the expression.

Abnormalities in Müller glial cells were also observed during inflammation. AT1R signaling promoted GFAP expression representing reactive Müller glial cells. The possible changes in reactive Müller glial cells included decreased uptake of glutamate<sup>47,48</sup> and GABA,<sup>47,49</sup> and they induced gliogenetic changes in the cells themselves afterward.<sup>50</sup> The accumulation of glutamate has been shown by clinical data in diabetic retinopathy.<sup>47</sup> These abnormalities might have affected the micro-environment of surrounding retinal neurons that indirectly altered their status.

Moreover, the changes in ERG responses were obvious. Telmisartan successfully preserved retinal function. Thus, AT1R signaling was responsible for disturbing visual function. The change in rhodopsin expression might have been involved in a-wave changes, and the malfunction of postsynaptic neural activity in INL cells or Müller glial cells might have been reflected in b-wave responses in ERG, though several other factors might also have been involved. These results further encourage us to use this therapy for patients with inflammatory diseases.

We demonstrated that local angiotensin II expression was extremely elevated during retinal inflammation, thereby influencing the condition of the retinal neural cells through AT1R signaling, which is expressed in most of the retinal cells. These changes were accelerated by positive feedback regulation through AT1R. Thus, telmisartan was effective for keeping the retinal neural cells from losing their physiological activities and normal ERG responses. We concluded that telmisartan plays key roles in neuroprotection and that it preserves good visual function by reducing inflammatory reactions in the retinal neural cells.

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