Selective Suppression of Pathologic, but Not Physiologic, Retinal Neovascularization by Blocking the Angiotensin II Type 1 Receptor

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PURPOSE. To investigate the anti-inflammatory and anti-angiogenic effects of telmisartan, an angiotensin II type 1 receptor (AT1-R) antagonist, on ischemia-induced retinal neovascularization.

METHODS. C57BL/6 neonatal mice were reared in an 80% concentration of oxygen from postnatal day (P7) to P12, followed by room-air breathing until P17, to induce ischemia-initiated retinal neovascularization (i.e., a murine model of ischemic retinopathy). Tissue localization of AT1-R was examined by immunohistochemistry for murine retinal wholemounts and human fibrovascular tissues excised at vitrectomy for proliferative diabetic retinopathy. Animals received intraperitoneal injection of telmisartan or vehicle. A concanavalin A lectin perfusion-labeling technique was used to evaluate the areas of physiological and pathologic retinal new vessels and the number of leukocytes adhering to the vasculature. Retinal mRNA and protein levels of intercellular adhesion molecule (ICAM)-1, vascular endothelial growth factor receptor (VEGFR)-1, and VEGFR-2 were examined by RT-PCR and ELISA.

RESULTS. Vessels in human fibrovascular tissues and the murine retinas were positive for AT1-R. Pathologic (P < 0.01), but not physiologic (P > 0.05), retinal neovascularization was significantly suppressed in telmisartan-treated mice compared with vehicle-treated animals. The number of adherent leukocytes (P < 0.01) was also significantly reduced, together with retinal ICAM-1 levels (P < 0.01) in the telmisartan-treated group compared with the control group. No significant difference was detected in retinal VEGFR-2 levels between the two groups, whereas retinal VEGFR-1 levels in the telmisartan-treated group were significantly (P < 0.05) lower than in the vehicle-treated group.

CONCLUSIONS. The present findings suggest that the AT1-R signaling blockade leads to the selective suppression of pathologic, but not physiological, retinal neovascularization through the inhibition of the inflammatory processes related to pathologic neovascularization. (Invest Ophthalmol Vis Sci. 2005;46:1078–1084) DOI:10.1167/iovs.04-1101
type of retinal neovascularization in a murine model of ischemic retinopathy.

**METHODS**

**Murine Model of Ischemic Retinopathy**

C57BL/6 mice (SLC, Shizuoka, Japan) were used. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The ethics committee of our institution approved all surgical interventions and animal care procedures, which were in accordance with the Guidelines and Policies for Animal Surgery provided by the Animal Study Committees of the Central Institute for Experimental Animals of Keio University. Postnatal day (P)7 mice with their nursing mothers were maintained for a full 5 days in 80% oxygen to generate the nonvascular retinal area, as described previously.25 On P12, they were placed in normoxia for an additional 5 days to induce retinal neovascularization.

**Intraperitoneal Injection of Telmisartan**

After 80% oxygen exposure, pups were categorized into three groups: The first had no treatment (ischemic retinopathy) and the other two received 0.1-ml intraperitoneal injections of vehicle or telmisartan for 5 days in normoxia (21% oxygen) after the hyperoxic exposure (P12-P16). Telmisartan was a gift of Boehringer Ingelheim (Ingelheim, Germany). We dissolved telmisartan in dimethyl sulfoxide (DMSO) in 30 mM diluted to 60 mM with phosphate-buffered saline (PBS) and injected at 3 μg/g body weight. This dose was sufficient to block AT1-R signaling to decrease systemic blood pressure in rats.26 The degree of retinal neovascularization and the number of adherent leukocytes were evaluated on P17.

**Lectin Labeling of Retinal Vasculature and Adherent Leukocytes**

The retinal vasculature and adherent leukocytes were imaged by perfusion-labelling with fluorescein-isothiocyanate (FITC)-coupled concanavalin A lectin (Con A; Vector, Burlingame, CA), as described previously.27 After deep anesthesia, the chest cavity was opened and a 27-gauge cannula was introduced into the left ventricle. After injection of 2 ml of PBS to remove erythrocytes and nonadherent leukocytes, 2 ml FITC-conjugated con A lectin was perfused. After the eyes were enucleated, the retinas were flatmounted. The flatmounts were imaged by epifluorescence microscope (IX71; Olympus, Tokyo, Japan), and area ratios of pathologic and physiological vessels. The results were processed for statistical analyses (Mann-Whitney test). Differences were considered statistically significant at P < 0.05.

**RT-PCR for Intercellular Adhesion Molecule-1 and Vascular Endothelial Growth Factor Receptor-1 and -2**

Total RNA was isolated from the retina using extraction reagent (Iso-gen; Nippon Gene, Toyama, Japan) and reverse-transcribed with a cDNA synthesis kit (First-Strand; Pharmacia Biotech, Uppsala, Sweden) according to the manufacturers’ protocols. PCR was performed with Taq DNA polymerase (Toyobo, Tokyo, Japan) in a thermal controller (MiniCycler; MJ Research, Watertown, MA). The primer sequences are as follows: 5'-ATG TGG CAC CAC ACC TCT TAC AAT GAG CTG CG-3' (sense) and 5'-CAT ACT CCT GCT TGC TGA TCC AGA TCT GC-3' (antisense; 37 bp) for β-actin, 5'-ATG TCG AGC TTT GGG ATG GTA-3' (sense) and 5'-CTG GCC TTG GAG ACT CAG TG-3' (antisense; 505 bp) for mouse intercellular adhesion molecule (ICAM)-1. Human/mouse vascular endothelial growth factor receptor-1 (VEGF R1) primers (302 bp; PCR Primer Pair; R&D Systems, Inc., Minneapolis, MN) and human/mouse VEGF R2 primers (569 bp; PCR Primer Pair; R&D Systems, Inc.) were used for VEGF-R1 and -2, respectively.

**ELISA for ICAM-1 and VEGFR-1 and -2**

The animals were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retina was carefully isolated and placed into 200 μl lysis buffer (0.02 M HEPES, 10% glycerol, 10 mM Na3PO4, 100 μM NaVO4, 1% Triton, 100 mM NaF, 4 mM EDTA [pH 8.0]) supplemented with protease inhibitors and sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C, and the ICAM-1 and VEGFR-1 and -2 levels in the supernatant were determined with mouse ICAM-1 and VEGFR-1 and -2 kits (Techno Corp., Minneapolis, MN) according to the manufacturer’s protocol. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration.

**Morphometric and Statistical Analyses**

All results are expressed as the mean ± SD. The number of leukocytes in each flat mount was counted independently by two investigators under the epifluorescence microscope. Area ratios of pathologic and physiological neovascularization to the flattened retina were measured and calculated. The morphology of the pathologic neovascularization was readily discerned from the intraretinal extension of physiological vessels. The results were processed for statistical analyses (Mann-Whitney test). Differences were considered statistically significant at P < 0.05.

**RESULTS**

**Localization of AT1-R in the Murine Retina and Human Fibrovascular Tissues**

immunohistochemistry for AT1-R was performed to identify its expression on the fibrovascular tissues of patients with proliferative diabetic retinopathy and on the murine retina. In the fibrovascular tissues, the vascular endothelium was positive for AT1-R (Figs. 1A, 1B). In the retina of neonatal mice, the AT1-R immunoreactivity was detected mainly on the endothelial cells of inner retinal vessels (Figs. 1C, 1D). Immunohistochemistry
Neovascularization and Inflammation

The retinal vasculature and adherent leukocytes were imaged by perfusion-labeling with FITC-coupled Con A. In normal P17 mice, the retina was covered with physiological (intraretinal) vessels, corresponding to the staining of PECAM-1, which is widely used as a marker of vascular endothelial cells (Figs. 1E–G).

Effects of Telmisartan on Pathologic Retinal Neovascularization and Inflammation

The retinal vasculature and adherent leukocytes were imaged by perfusion-labeling with FITC-coupled Con A. In normal P17 mice, the retina was covered with physiological (intraretinal) vessels, corresponding to the staining of PECAM-1, which is widely used as a marker of vascular endothelial cells (Figs. 1E–G).

Leukocyte counts showed a significant \( (P < 0.01) \) decrease in the telmisartan group \((21.3 \pm 1.3\%\) \) compared with the vehicle group \((53.7 \pm 9.4\%\) \) or the nontreated ischemic retinopathy group \((55.2 \pm 5.7\%\) \).

Effects of Telmisartan on Physiological Retinal Neovascularization

Area ratios of physiological neovascularization in ischemic retinopathy were compared among nontreated, vehicle- and telmisartan-treated groups (Figs. 2B, 2E, 2F). No significant \( (P > 0.05) \) difference was detected in physiological neovascularization area between the telmisartan group \((62.7 \pm 0.8\%\) \) and the vehicle group \((55.2 \pm 0.7\%\) \) or the nontreated retinopathy group \((69.9 \pm 4.4\%\) \). To confirm further this sparing effect of telmisartan on physiological vasculature, normal adult mice (Figs. 5A, 5B) and neonates undergoing retinal vascular development (Figs. 5C, 5D) were used. There was no morphologic difference in adult physiological vessels between vehicle-treated (Fig. 5A) and telmisartan-treated (Fig. 5B) groups. As for physiological neovascularization during postnatal retinal development, no significant \( (P > 0.05) \) difference was detected in physiological neovascularization area between the telmisartan group \((98.8 \pm 2.4\%\) \) and the...
vehicle group (99.8% ± 0.9%) or the nontreated group (99.7% ± 0.9%).

Detection of mRNA and Protein Levels Of ICAM-1 and VEGFR-1 and -2
Retinas from P17 mice were subjected to RT-PCR and ELISA analyses to detect the mRNA and protein levels of ICAM-1, VEGFR-1, and VEGFR-2 (Fig. 6). Retinal ICAM-1 mRNA levels in ischemic retinopathy treated with vehicle were higher than in normal age-matched mice. Systemic administration of telmisartan substantially reduced ICAM-1 mRNA levels (Fig. 6A). Similarly, retinal ICAM-1 protein levels were significantly attenuated by the treatment with telmisartan (P < 0.01, Fig. 6B).

Retinal VEGFR-1 mRNA and protein levels in vehicle-treated mice with retinopathy tended to increase compared with those in normal age-matched mice, but the difference was not statistically significant (P > 0.05). Telmisartan treatment for ischemic retinopathy significantly attenuated both mRNA and protein levels of retinal VEGFR-1 (P < 0.05; Figs. 6A, 6C). In contrast, there was no difference in retinal VEGFR-2 mRNA or protein levels among normal age-matched mice and the vehicle group (99.8% ± 0.9%) or the nontreated group (99.7% ± 0.9%).

FIGURE 3. Effects of telmisartan on pathologic neovascularization (A) and inflammation (B). Telmisartan-treated mice with retinopathy showed significantly less pathologic neovascularization and significantly fewer adherent leukocytes than nontreated or vehicle-treated mice. The results represent means ± SD. *P < 0.01 by Mann-Whitney test.

FIGURE 4. Effects of telmisartan on physiological neovascularization in ischemic retinopathy (A) and during normal vascular development (B). Telmisartan-treated mice exhibited no significant difference in physiological neovascularization compared with vehicle-treated or nontreated mice. The results represent means ± SD. *P < 0.01 by Mann-Whitney test.

DISCUSSION
The present study demonstrates for the first time that AT1-R blockade selectively prevents pathologic retinal neovascularization, while sparing physiological neovascularization that compensates for the preceding retinal ischemia. Furthermore, we showed that the mechanisms in the differential modulation of retinal neovascularization depend, at least in part, on the suppression of ICAM-1-mediated leukocyte adhesion to the retinal vasculature, inflammatory processes that characterize pathologic retinal neovascularization.

We have proposed that ischemia-induced retinal neovascularization, when it becomes pathologic, involves inflammatory processes.18–20 A previous immunohistochemical study pointed out the infiltration of macrophages in fibrovascular tissues excised at vitrectomy for proliferative diabetic retinop-
athy, indicating a possible link between retinal neovascularization and inflammation. In an animal model of ischemic retinopathy, pathologic, but not physiological, neovascularization was shown to be preceded and accompanied by the adhesion of inflammatory monocytes to the retinal vasculature. When clodronate-liposome, a reagent that induces apoptosis specifically in monocyte/macrophage cells, was used, pathologic retinal neovascularization was suppressed without any substantial effects on physiological neovascularization. In addition, other reports have suggested the proangiogenic role of inflammatory monocytes and macrophages in murine ischemic retinopathy. Intravitreally infiltrating macrophages adjacent to the pathologic new vessels express and produce VEGF in the animal model. Neutralizing antibodies against monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α have been shown to reduce pathologic retinal neovascularization and inflammation. Therefore, inflammatory monocytes are likely to disrupt the direction of physiological neovascularization, triggering pathologic retinal neovascularization.

ICAM-1 is a ligand for β2-integrins constitutively expressed on the leukocyte surface and is a key adhesion molecule that controls leukocyte adhesion to the vessel walls. ICAM-1, constitutively expressed on vascular endothelial cells at a low level, is swiftly upregulated during inflammation, resulting in enhancement of leukocyte-endothelial interaction. A previous study in donor eyes demonstrated that diabetic retinas had increased levels of ICAM-1 immunoreactivity in the vessels as well as infiltrating leukocytes, compared with normal retinas. In a rodent model of diabetes, ICAM-1-dependent leukocyte adhesion is enhanced in the early stage, and various retinal diseases related to long-term diabetes have been shown to be mediated by ICAM-1. In vitro, Ang II was shown to induce the expression of ICAM-1 on vascular endothelial and promote leukocyte-endothelial adhesion.

![Figure 5](image1.png)

**FIGURE 5.** Flatmounted retinas from normal adult mice (A, B) and neonates with physiological vascular development (C, D). No morphologic difference was detected between vehicle-treated (A, C) and telmisartan-treated mice (B, D).

![Figure 6](image2.png)

**FIGURE 6.** RT-PCR (A) and ELISA (B, C) analyses for retinal ICAM-1 and VEGFR-1 and -2. (A) Retinal ICAM-1 mRNA expression in ischemic retinopathy treated with telmisartan, reduced to those in normal mice, was lower than those in vehicle-treated mice with retinopathy. Retinal VEGFR-1 mRNA expression in telmisartan-treated retinopathy mice also decreased compared with vehicle-treated mice with retinopathy. There was no apparent difference in retinal VEGFR-2 expression between vehicle-treated and telmisartan-treated mice. (B) Retinal ICAM-1 protein levels in vehicle-treated mice with retinopathy were significantly higher than in normal mice and were significantly suppressed by treatment with telmisartan. The results are expressed as the mean ± SD; n = 6. *P < 0.01 by Mann-Whitney test. (C) Retinal VEGFR-1 protein levels in telmisartan-treated mice with retinopathy were significantly higher than in vehicle-treated mice with retinopathy; n = 6. †P < 0.05. (D) No significant (P > 0.05) difference in retinal protein levels of VEGFR-2 was detected among three groups (n = 6).
vitro data, telmisartan-treated animals in our study exhibited the decreased retinal ICAM-1 mRNA and protein expression as well as the suppressed leukocyte adhesion to the retinal vessels, reasonably resulting in the inhibition of inflammation-related pathologic neovascularization.

As for other proangiogenic mechanisms shown in the literature, Ang II induced the in vitro expression of VEGFR-2 mRNA on cultured bovine retinal endothelial cells and pericytes, enhancing VEGF-induced angiogenic activity.\textsuperscript{19,16} VEGF has two cognate receptors called VEGFR-1 and -2.\textsuperscript{36–38} VEGF-mediated endothelial cell mitogenic activity was shown to depend, not on VEGFR-1, but on VEGFR-2.\textsuperscript{39} In our ischemic retinopathy model, as others reported,\textsuperscript{40} retinal VEGFR-2 expression showed levels similar to those in retinal vascular development, and telmisartan had a minimal effect on VEGFR-2 expression levels in vivo. Although VEGFR-2 blockade in the retinopathy model was reported to result in the suppression of pathologic neovascularization,\textsuperscript{40} the telmisartan-induced effects shown in the present study are thought to be mediated, not by VEGFR-2 inhibition, but mainly by ICAM-1 inhibition, causing the prevention of inflammation to maintain the right direction of physiological neovascularization.

The current data show that AT1-R blockade led to the significant decrease in retinal VEGFR-1 levels in ischemic retinopathy. Shih et al.\textsuperscript{41} showed that VEGFR-1 signaling activated by placental growth factor (PIGF)-1 led to the suppression of hyperoxia-induced vaso-obliteration and suggested the possibility of VEGFR-1-mediated prevention of pathologic retinal neovascularization secondary to the decreased extent of retinal ischemia. They also described that PIGF-activated signaling of VEGFR-1 did not affect any of three types of vaso-proliferation (i.e., physiological neovascularization during normal retinal development, physiological neovascularization after hyperoxia-induced ischemia, or pathologic neovascularization after hyperoxia-induced ischemia). In the present study, we applied telmisartan to mice with retinopathy during the proliferative stage after the phase of hyperoxia-induced vaso-obliteration. Reasonably, our administration of telmisartan did not affect the extent of avascular area formation in these mice. Because vasoproliferation after the ischemic phase depends not on VEGFR-1,\textsuperscript{41} but on VEGFR-2,\textsuperscript{42} VEGFR-1 downregulation on vascular endothelial cells is thought to have little or no effect on retinal neovascularization. In contrast, VEGFR-1 is well-known to be expressed on inflammatory leukocytes including monocytes.\textsuperscript{42–44} The telmisartan-induced decrease in retinal VEGFR-1 seen in the present study, therefore, is compatible with and explained at least in part by the suppression of VEGFR-1-bearing inflammatory leukocytes' adhering to the retinal vasculature. Although in a recent paper the antiangiogenic effect of AT1-R blockade (losartan) on pathologic retinal neovascularization in ischemic retinopathy was reported,\textsuperscript{45} no comparison was made between physiological versus pathologic neovascularization, and no mechanistic explanation was provided about the inhibitory effects on pathologic neovascularization. In this context, the present study is the first to indicate the molecular and cellular mechanisms in the differential effects of AT1-R blockade on both types of retinal neovascularization. Our novel findings are supported by a recent paper showing that Ang II-mediated subcutaneous neovascularization in a murine model (Matrigel; BD Biosciences, Franklin Lakes, NJ) is mediated by inflammation-related pathways.\textsuperscript{6}

Currently, several AT1-R antagonists are widely applied to hypertensive patients and have been shown to be safe and effective.\textsuperscript{45,46} Hypertension is well known to be a worsening factor in proliferative diabetic retinopathy.\textsuperscript{47} A recent report\textsuperscript{48} has shown the increased levels of Ang II in the vitreous fluid of patients with proliferative diabetic retinopathy, although its role in pathogenesis remains undetermined. More work is needed to establish the validity of this therapeutic approach in vision-threatening ischemic retinopathies.

**References**


