Suppression of Diabetes-Induced Retinal Inflammation by Blocking the Angiotensin II Type 1 Receptor or Its Downstream Nuclear Factor-κB Pathway

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Purpose. To investigate the involvement of the renin-angiotensin system (RAS) and the nuclear factor (NF)-κB pathway with diabetes-induced retinal inflammation.

Methods. Six weeks after induction of diabetes, C57BL/6 mice were treated with the angiotensin II type 1 receptor (AT1-R) blocker (ARB) telmisartan or valsartan, the AT2-R blocker PD123319, or the NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) daily for 1 week. Retinal mRNA and protein levels of the RAS components were examined by RT-PCR and Western blot, respectively. Leukocyte adhesion to the retinal vasculature was evaluated with a concanavalin A lectin perfusion-labeling technique. Retinal expression levels of intercellular adhesion molecule (ICAM)-1 and vascular endothelial growth factor (VEGF) were examined by RT-PCR and ELISA. ARB or DHMEQ was applied to murine capillary endothelial (b-End3) cells stimulated with a high concentration of glucose to analyze nuclear translocation of NF-κB via immunohistochemistry for p65 and mRNA and protein levels of ICAM-1 and monocyte chemotactic protein (MCP)-1.

Results. Induction of diabetes led to a significant increase in retinal expression and production of the RAS components including angiotensin II, AT1-R, and AT2-R. Retinal adherent leukocytes were significantly suppressed by AT1-R, but not by AT2-R, blockade. Administration of the ARB, but not of PD123319, inhibited diabetes-induced retinal expression of ICAM-1 and VEGF. DHMEQ also suppressed these cellular and molecular inflammatory parameters in the diabetic retina to the levels obtained with ARB treatment. In vitro, glucose-induced nuclear translocation of NF-κB p65 and upregulation of ICAM-1 and MCP-1 were significantly suppressed by application of the ARB. The in vivo treatment with the ARB, as well as DHMEQ, attenuated the diabetes-induced retinal expression of angiotensin II and AT1-R, per se.

Conclusions. The present data revealed significant a contribution of the AT1-R/NF-κB pathway to diabetes-induced retinal inflammation, providing a mechanistic reason for targeting AT1-R or NF-κB in the treatment of diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2007;48:4342–4350) DOI:10.1167/iovs.06-1473

Diabetic retinopathy is a significant cause of severe vision loss and blindness in working-age adults.[1] Increasing evidence suggests that the pathogenesis of diabetic retinopathy is mediated by inflammatory processes, including leukocyte adhesion and the cytokine network.[2–7] Retinal vasculature in diabetes is accompanied by inflammatory cell adhesion,[8] which triggers vascular hyperpermeability[9] and pathologic retinal neovascularization.[2] Clinically, elevated levels of vascular endothelial growth factor (VEGF)[10] and monocyte chemotactic protein (MCP)-1[11] were detected in the vitreous fluid of eyes with proliferative diabetic retinopathy. Enhanced immunoreactivity of intercellular adhesion molecule (ICAM)-1 in retinal vessels, together with leukocyte infiltration, was observed in donor eyes with diabetic retinopathy.[12]

The renin–angiotensin system (RAS) plays an important role in the regulation of systemic blood pressure. Angiotensin II, the final product of the system working as a constrictor of blood vessels, has two cognate receptors, angiotensin II type 1 receptor (AT1-R) and AT2-R.[13] Because the major pathogenic signaling of angiotensin II is mediated by AT1-R, AT1-R blockers (ARBs) are widely used in patients with hypertension and cardiovascular diseases. Recently, various functions of the RAS have been pointed out, including angiogenesis[14,15] and inflammation.[16] We have shown the inhibitory effect of ARBs on several retinal disorders mediated by ICAM-1, including ischemia-induced retinal neovascularization,[17] endotoxin-induced retinal inflammation,[18] and choroidal neovascularization.[19]

Nuclear factor (NF)-κB is a transcription factor activated by various stimuli such as bacterial endotoxins, inflammatory cytokines, hypoxia, and hyperglycemia, and it plays a critical role in the regulation of gene expression of inflammation-related molecules including adhesion molecules, chemokines, and cytokines.[20] AT1-R downstream signaling is known to lead to the activation of NF-κB.[21] In alloxan-induced diabetic rats, NF-κB activation was shown to be an early retinal event, possibly linked to the development of diabetic retinopathy.[22]

Recent clinical and experimental studies have indicated the association of the RAS with diabetic retinopathy. Clinically, angiotensin-converting enzyme (ACE) inhibition results in significant suppression of the progression of retinopathy in normotensive subjects with type 1 diabetes.[23] However, molecu-

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TABLE 1. Sequences of PCR Primers

Molecule		Primer Sequences	Product Size (bp)
ICAM-1	Sense	5′-GTG TCG AGC TTT GGG ATG GTA-3′	505
	Antisense	5′-CTG GGC TTG GAG ACT CAG TG-3′	
VEGF	Sense	5′-GAA GTC CCA TGA AGT GAT CCA G-3′	319 for VEGF120
	Antisense	5′-TCA CCG CCT TGG CTT GTC A-3′	451 for VEGF164
MCP-1	Sense	5′-CCC CAC TCA CCT GCT GCT ACT-3′	380
	Antisense	5′-GGC ATC ACA GTC CGA GTC ACA-3′	
AT1-R	Sense	5′-TCA CCT GCA TCA TCA TCT GG-3′	204
	Antisense	5′-AGC TGG TAA GAA TGA TTA-GG-3′	
AT2-R	Sense	5′-CCA GCA GCC GTC CTT TTG ATA A-3′	679
	Antisense	5′-GTA ATT CTG TTC TTC CCA TAG C-3′	
Angiotensinogen	Sense	5′-CAC CCC TGC TAC AGT CCA TTG-3′	231
	Antisense	5′-GTC TGT ACT GAC CCC CTC-3′	
β-Actin	Sense	5′-ATG TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′	837
	Antisense	5′-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3′	

lar and cellular mechanisms by which the RAS plays a role in the pathogenesis of diabetic retinopathy remain to be elucidated, although recent in vivo experiments have demonstrated that inhibition of the RAS leads to significant amelioration of vascular hyperpermeability[24] and blood flow[25] in the diabetic retina. Herein, we report the in vivo and in vitro suppression of diabetes-induced retinal inflammation and glucose-stimulated endothelial reaction by blocking AT1-R or its downstream mediator NF-κB, together with underlying molecular and cellular mechanisms.

METHODS

Animals and Induction of Diabetes

Male C57BL/6 mice (CLEA, Tokyo, Japan) at the age of 6 to 8 weeks were used. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 mice received an intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich St. Louis, MO) at a dose of 60 mg/kg body weight (BW). Animals with plasma glucose levels greater than 250 mg/dL 7 days after injection were considered diabetic.

Blockade of AT1-R, AT2-R, and NF-κB

Six weeks after induction of diabetes, animals were treated with an ARB (telmisartan or valsartan), an AT2-R blocker (PD123319; Sigma-Aldrich), an NF-κB inhibitor (DHMEQ; dehydroxymethylepoxyquinomicin), or vehicle (0.25% dimethyl sulfoxide [DMSO] in phosphate-buffered saline [PBS]) daily for 1 week. ARBs and DHMEQ were injected into mice intraperitoneally. PD123319 was administered by subcutaneous osmotic minipump (Alzet model 2001; Alza, Palo Alto, CA), as described previously.[26] Telmisartan and valsartan were kind gifts from Boehringer Ingelheim (Ingelheim, Germany) and Novartis Pharma (Basel, Switzerland), respectively. DHMEQ is a novel NF-κB inhibitor, based on the structure of epoxyquinomicin C, which was originally isolated from *Amycolatopsis*.[27] DHMEQ has been shown to inhibit nuclear translocation of NF-κB without affecting phosphorylation and degradation of I-κBα. Optically active (−)-DHMEQ[28] was used throughout the experiments. Mice received telmisartan at a dose of 0.5 or 5 mg/kg BW, valsartan at 1 or 10 mg/kg BW, PD123319 at 1 or 10 mg/kg BW, or DHMEQ at 1 or 5 mg/kg BW. Systolic blood pressure was assessed by a computerized, noninvasive tail cuff system (MK-2000; Muromachi Kikai, Tokyo, Japan) after each 1-week treatment.

Quantification of Retinal Adherent Leukocytes

The retinal vasculature and adherent leukocytes were imaged by perfusion-labeling with fluorescein-isothiocyanate (FITC)-coupled concanavalin A lectin (Con A) (Vector, Burlingame, CA), as described previously.[7] With the mouse under 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 of FITC-conjugated Con A was perfused. After the eyes were enucleated, the retinas were flatmounted. The flatmounts were imaged with an epifluorescence microscope (IX71; Olympus, Tokyo, Japan), and the total number of Con A-stained adherent leukocytes per retina was determined.

Western Blot Analyses for AT1-R, AT2-R, and Angiotensin II

Animals were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retina was carefully isolated and placed into 100 μL of lysis buffer (0.02 M HEPES, 10% glycerol, 10 mM $Na_4P_2O_7$, 100 μM Na_3VO_4, 1% Triton, 100 mM NaF, 4 mM EDTA [pH 8.0]) supplemented with protease inhibitors (2 mg/L aprotinin, 100 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 2.5 μM pepstatin A) and sonicated. The lysate was centrifuged and the supernatant was collected. Each sample containing 30 μg of total protein was separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), and electroblotted to PVDF (polyvinylidene fluoride) membranes (ATTO, Tokyo, Japan). After nonspecific binding was blocked with 5% skim milk, the membranes were incubated with a rabbit polyclonal antibody against angiotensin II (1:200), AT1-R (1:100), or AT2-R (1:300; Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-α-tubulin antibody (1:2000; Sigma-Aldrich) at 4°C overnight, followed by incubation with a horseradish peroxidase–conjugated goat antibody against rabbit IgG (1:5000; BioSource, Camarillo, CA). The signals were visualized with chemiluminescence (ECL kit; GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol.

RT-PCR Analyses for ICAM-1, VEGF, MCP-1, AT1-R, AT2-R, and Angiotensinogen

Total RNA was isolated from the retina using an extraction reagent (TRIzol; Invitrogen, Carlsbad, CA) and reverse-transcribed with a cDNA synthesis kit (First-Strand; GE Healthcare). PCR was performed with *Taq* DNA polymerase (Takara Bio, Ohtsu, Japan) in a thermal controller (Gene Amp PCR system; Applied Biosystems, Foster, CA). The primer sequences and the expected size of amplified cDNA fragments are indicated in Table 1.

Enzyme-Linked Immunosorbent Assay (ELISA) for ICAM-1 and VEGF

The retina was carefully isolated from the eyes after the treatments and placed into 100 μL of lysis buffer supplemented with protease inhibitors and sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C, and the protein levels of ICAM-1 and VEGF were determined with the mouse ICAM-1 and VEGF ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols.

TABLE 2. Systemic Data

	Normal (n = 20)	Diabetes				
		Vehicle (n = 23)	Telmisartan (5 mg/kg) (n = 22)	Valsartan (10 mg/kg) (n = 22)	PD123319 (10 mg/kg) (n = 21)	DHMEQ (5 mg/kg) (n = 21)
Body weight (g)	27.7 ± 2.3	23.0 ± 3.7*	24.2 ± 3.2*	23.8 ± 3.1*	23.8 ± 2.8*	23.0 ± 3.5*
Plasma glucose (mg/dL)	159 ± 23	467 ± 135*	463 ± 136*	446 ± 132*	463 ± 170*	451 ± 127*
Systolic blood pressure (mm Hg)	122 ± 14	123 ± 11	107 ± 9†	108 ± 15†	127 ± 14	123 ± 12

* $P < 0.01$ compared with normal.
† $P < 0.05$ compared with vehicle.

In Vitro Assays

Murine brain-derived capillary endothelial cells (b-End3) were cultured with DMEM (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) and 5.5 mM D-glucose at 37°C in a 95% air-5% CO_2 atmosphere. After the cells achieved confluence, they were incubated with the same medium containing normal (5.5 mM) or high (25 mM) concentration of D-glucose alone, high glucose plus telmisartan (3 μM), high glucose plus valsartan (3 μM), or high glucose plus DHMEQ (10 μg/mL) for 12 hours. To exclude bias from the effects of hyperosmolarity, the cells were incubated in a medium containing 5.5 mM D-glucose supplemented with 19.5 mM mannitol. Total cellular RNA from b-End3 cells was isolated and PCR was performed to evaluate mRNA expression of ICAM-1 and MCP-1. For protein analyses, supernatant and cell lysates were collected after a 24-hour incubation, and then the concentrations of MCP-1 in the supernatant and ICAM-1 in the cell lysates were measured by the ELISA kits (R&D Systems).

Immunocytochemistry for NF-κB p65

The b-End3 cells were incubated with the culture medium containing a normal (5.5 mM) or high (25 mM) concentration of D-glucose alone, high glucose plus telmisartan (3 μM), high glucose plus valsartan (3 μM), or high glucose plus DHMEQ (10 μg/mL) for 24 hours. After incubation, cells were fixed in methanol and washed with PBS. Nonspecific binding was blocked by incubating the cells for 1 hour in PBS containing 3% normal goat serum and 0.4% Triton. These cells were then incubated with a rabbit polyclonal antibody against human NF-κB p65 (1:100, Santa Cruz Biotechnology) overnight at 4°C. Cells were subsequently washed and incubated with an avidin-Alexa 488–tagged secondary antibody (1:200; Invitrogen-Molecular Probes, Eugene, OR) for 40 minutes at room temperature. For nuclear staining, the cells were treated with TOTO-3 (1:500; Invitrogen-Molecular Probes). After two washes, the cells were viewed with the epifluorescence microscope (Olympus).

Morphometric and Statistical Analyses

All results are expressed as mean ± SD. The values were processed for statistical analyses (Mann-Whitney test), and differences were considered statistically significant at $P < 0.05$.

RESULTS

Metabolic and Hemodynamic Parameters

Diabetic mice showed a significant ($P < 0.01$) decrease in body weight and a significant ($P < 0.01$) increase in blood glucose, compared with age-matched normal controls (Table 2). Treatment with telmisartan, valsartan, PD123319, or DHMEQ did not significantly change these metabolic parameters. Compared with vehicle-treated diabetic animals, telmisartan and valsartan, but not PD123319 or DHMEQ, significantly ($P < 0.05$) reduced systolic blood pressure in diabetic mice.

Upregulation of the RAS Components in the Diabetic Retina

The murine retina was subjected to RT-PCR and Western blot analyses to detect the expression of the RAS components at the mRNA (Fig. 1A) and protein (Figs. 1B–E) levels, respectively. Angiotensinogen, AT1-R, and AT2-R expression was substantially higher in the diabetic retina than in the retina from age-matched normal control mice (Fig. 1A). Similarly, angiotensin II (Fig. 1C), AT1-R (Fig. 1D), and AT2-R (Fig. 1E) protein levels were significantly upregulated by induced diabetes ($P < 0.05$).

Suppression of Retinal Adherent Leukocytes by AT1-R, but Not AT2-R, Blockade

The retinal adherent leukocytes were imaged by perfusion labeling with FITC-coupled Con A. Leukocyte counts were evaluated

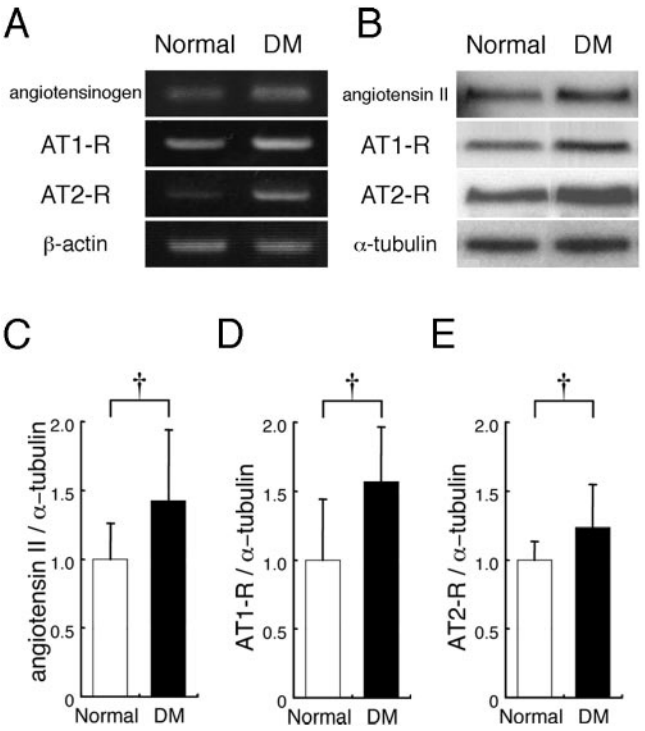

FIGURE 1. Upregulation of the retinal RAS components after induction of diabetes. RT-PCR (**A**) and Western blot (**B–E**) for expression and production of the RAS components in the retina. Retinal mRNA expression of angiotensinogen, AT1-R, and AT2-R was substantially higher in diabetic mice than in age-matched normal mice (**A**). Retinal protein levels of angiotensin II, AT1-R, and AT2-R were significantly upregulated by induced diabetes (**B–E**). Data are the ratio of angiotensin II (**C**), AT1-R (**D**), and AT2-R (**E**) to α-tubulin ($n = 6$; †$P < 0.05$).

in the whole retina from the posterior retina around the optic disc (Figs. 2A–E, left) to the peripheral (anterior) retina next to the ora serrata (Figs. 2A–E, right). The total number of adherent leukocytes was significantly ($P < 0.01$) higher in vehicle-treated diabetic mice (22.1 ± 6.6 cells, Fig. 2B) than in normal, age-matched control animals (5.6 ± 2.7 cells, Fig. 2A). Telmisartan-treated diabetic mice at a dose of 5 mg/kg (10.9 ± 3.1 cells, Fig. 2C) and valsartan-treated diabetic mice at a dose of 10 mg/kg (11.1 ± 2.7 cells, Fig. 2D) showed a significant ($P < 0.01$) decrease in the leukocyte count, compared with vehicle-treated diabetic mice (Fig. 2B). Administration of telmisartan or valsartan suppressed leukocyte adhesion in the diabetic retina in a dose-dependent fashion (Fig. 2F). In contrast, AT2-R blockade with PD123319 at the dose of 10 mg/kg (22.4 ± 1.9 cells, Fig. 2E) did not significantly ($P > 0.05$) change the number of retinal adherent leukocytes, compared with vehicle-treated diabetic mice (22.2 ± 5.1 cells, Fig. 2G).

Suppression of Retinal Expression of ICAM-1 and VEGF by AT1-R, but Not AT2-R, Blockade

AT1-R blockade by systemic administration of telmisartan (5 mg/kg) or valsartan (10 mg/kg) substantially reduced retinal mRNA expression of ICAM-1 and VEGF upregulated by inducing diabetes (Fig. 3A). Retinal protein levels of ICAM-1 (Fig. 3B) and VEGF (Fig. 3C) were significantly higher in vehicle-treated diabetic mice than in age-matched normal control animals. AT1-R blockade by telmisartan (5 mg/kg) or valsartan (10 mg/kg), but not AT2-R blockade with PD123319 (10 mg/kg), significantly suppressed retinal protein levels of these inflammatory molecules.

Suppression of Retinal Adherent Leukocytes and Retinal Inflammatory Molecules by NF-κB Inhibition

The number of retinal adherent leukocytes was significantly smaller in DHMEQ-treated diabetic mice at the dose of 5 mg/kg (7.8 ± 2.3 cells) than in vehicle-treated diabetic mice (22.1 ± 6.6 cells). NF-κB inhibition with DHMEQ significantly decreased adherent leukocytes in a dose-dependent fashion (Fig. 4A). The mRNA expression (Fig. 4B) and protein levels of ICAM-1 (Fig. 4C) and VEGF (Fig. 4D), upregulated by induction of diabetes, were significantly suppressed by systemic application of DHMEQ at a dose of 5 mg/kg. The NF-κB inhibition with DHMEQ showed equivalently ($P > 0.05$) suppressive effects on these diabetes-induced retinal inflammatory parameters compared with the ARB treatment (Figs. 2F, 3B, 3C).

In Vitro Suppression of Glucose-Induced NF-κB Activation and Inflammatory Molecules by AT1-R Blockade

Nuclear translocation of NF-κB p65 in b-End3 cells, enhanced by high glucose, was significantly suppressed by application of telmisartan or valsartan to the level seen in NF-κB inhibition with DHMEQ (Figs. 5A, 5B). The mRNA expression of ICAM-1 and MCP-1 in b-End3 cells, induced by the 12-hour exposure to high glucose, was substantially suppressed by treatment with telmisartan, valsartan, or DHMEQ (Fig. 5C). AT1-R blockade with telmisartan or valsartan, as well as NF-κB inhibition with DHMEQ, significantly reduced protein levels of ICAM-1 (Fig. 5D) and MCP-1 (Fig. 5E) upregulated by high glucose stimulation.

Suppression of Diabetes-Induced Retinal Upregulation of the RAS Components by AT1-R Blockade

Since AT1-R blockade for the tissue, but not circulatory, RAS had been reported to reduce the RAS components per se[29,30] in addition to several inflammatory molecules, we investigated the in vivo effect of AT1-R or NF-κB inhibition on the angiotensin II-AT1-R ligand-receptor system in the diabetic retina. AT1-R blockade by telmisartan (5 mg/kg) or NF-κB inhibition

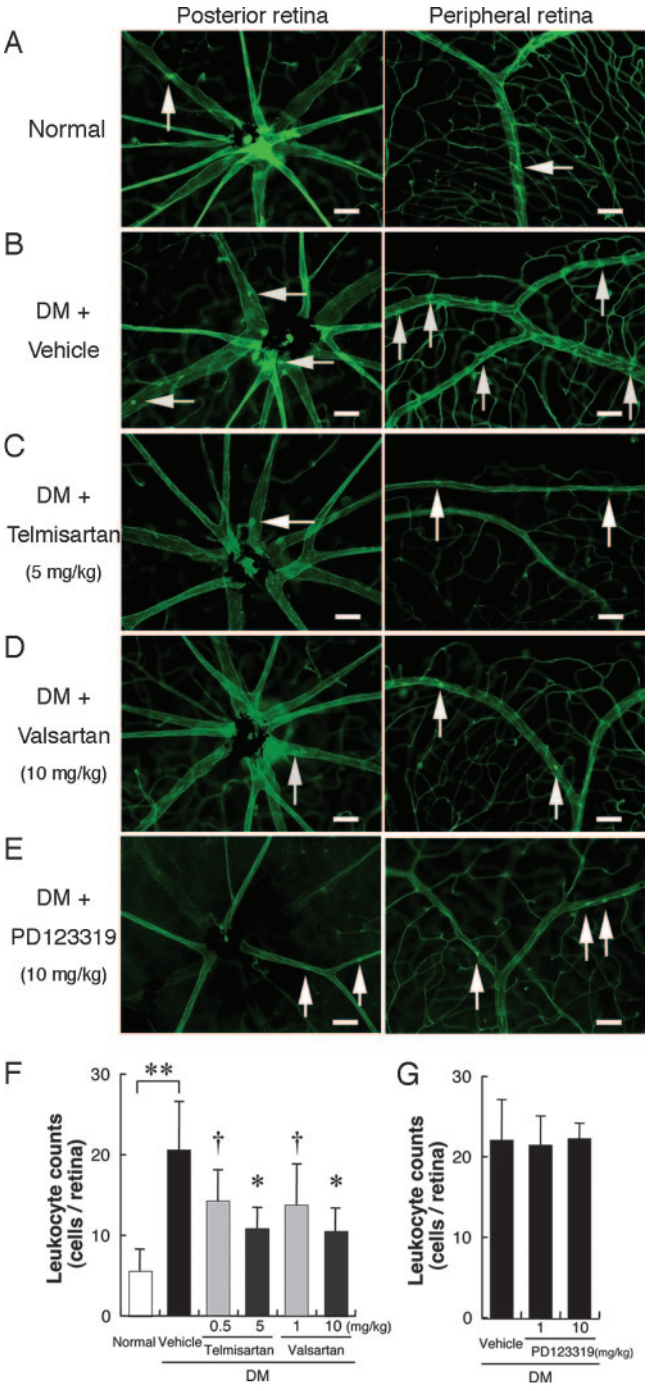

FIGURE 2. Inhibitory effect of AT1-R blockade on retinal adherent leukocytes. Flatmounted retinas from normal (**A**), vehicle-treated (**B**), telmisartan (5 mg/kg)-treated (**C**), valsartan (10 mg/kg)-treated (**D**), and PD123319 (10 mg/kg)-treated diabetic (**E**) mice. Vehicle-treated diabetic mice (**B**) showed an increased number of adherent leukocytes (*arrows*) compared with normal control mice (**A**). The treatment with telmisartan (**C**) or valsartan (**D**), but not PD123319 (**E**), decreased the number of adherent leukocytes. Scale bar, 50 μm. (**F**, **G**) The number of retinal adherent leukocytes. Telmisartan- or valsartan-treated diabetic mice had significantly fewer adherent leukocytes than did vehicle-treated diabetic mice. ($n = 15$; $^{**}P < 0.001$, $^{*}P < 0.01$, $\dagger P < 0.05$).

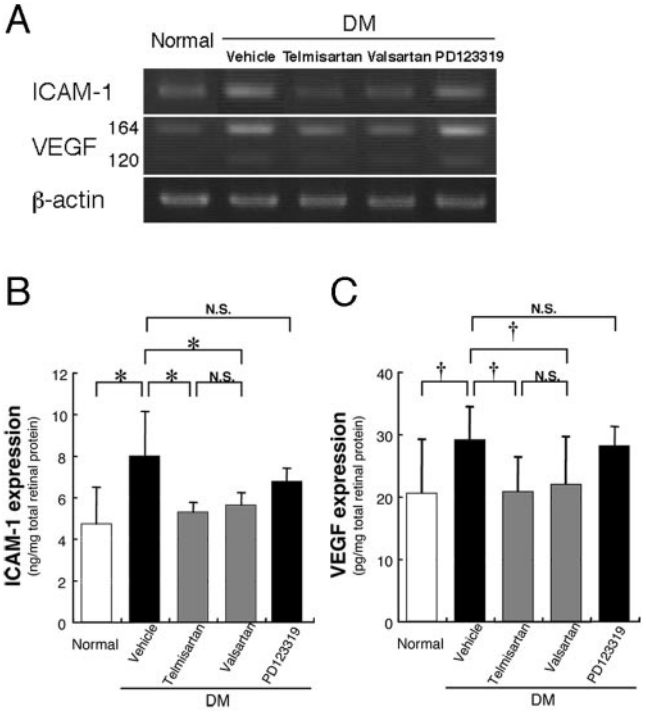

FIGURE 3. Inhibitory effects of AT1-R, but not AT2-R, blockade on retinal expression of inflammatory molecules analyzed by RT-PCR (**A**) and ELISA (**B**, **C**). (**A**) AT1-R blockade with telmisartan or valsartan substantially reduced mRNA expression of ICAM-1 and VEGF upregulated after induction of diabetes. (**B**, **C**) AT1-R blockade with telmisartan (5 mg/kg) or valsartan (10 mg/kg) significantly suppressed retinal protein levels of ICAM-1 (**B**) and VEGF (**C**) elevated after induction of diabetes. ($n = 8$; $^*P < 0.01$, $\dagger P < 0.05$).

with DHMEQ (5 mg/kg) substantially reduced retinal mRNA expression of angiotensinogen and AT1-R upregulated by inducing diabetes (Fig. 1A). Similarly, application of telmisartan (5 mg/kg) or DHMEQ (5 mg/kg) led to significant ($P < 0.01$) suppression of retinal angiotensin II and AT1-R protein levels.

DISCUSSION

The present study reveals, for the first time to our knowledge, several important findings concerning the relationship of the RAS and the NF-κB pathway with diabetes-induced retinal inflammation. First, the expression and production of the RAS-related molecules were upregulated in the retina when experimental diabetes was induced (Fig. 1). Second, diabetes-induced leukocyte adhesion to the retinal vasculature was shown to be suppressed by blocking AT1-R, but not AT2-R, signaling (Fig. 2). Third, the molecular mechanisms in the ARB-induced suppression of retinal leukocyte adhesion included the inhibitory effects on diabetes-induced expression of the inflammatory molecules VEGF and ICAM-1 (Fig. 3). The inhibition of NF-κB nuclear translocation exhibited equivalent effects on these diabetes-induced retinal inflammatory parameters compared with AT1-R blockade (Fig. 4). An important finding was that in vitro ARB application led to significant suppression of glucose-induced expression of ICAM-1 and MCP-1 via inhibiting NF-κB nuclear translocation (Fig. 5). In addition to its suppressive effect on these inflammatory parameters, the in vivo ARB or DHMEQ treatment attenuated diabetes-induced retinal expression of angiotensin II and AT1-R per se (Fig. 6).

Our data revealed the upregulation of the RAS components including angiotensin II, AT1-R, and AT2-R in the diabetic retina (Fig. 1). The circulatory RAS functions as a regulator of systemic blood pressure, whereas the tissue RAS is related to pathogenesis in several organs. In the retina as well, the RAS components proved to be present,[17,18,31–33] and the roles of the RAS in retinal neovascularization[17,31,33] and inflammation[18,32] were shown in recent in vivo studies, including ours. In vivo and in vitro studies demonstrated diabetes-induced upregulation of AT1-R expression in the kidney[34] and the pancreas[35] and glucose-induced upregulation of angiotensinogen in renal tubular cells.[36] Recent clinical laboratory data, in accordance with the present in vivo results (Fig. 1), revealed the elevated levels of angiotensin II in the vitreous samples from patients with proliferative diabetic retinopathy[37] and diabetic macular edema.[38]

Our blocking experiments revealed that diabetes-induced leukocyte adhesion to the retinal vasculature depended on angiotensin II signaling via AT1-R, but not AT2-R (Fig. 2). This is compatible with our recent reports showing the suppressive effect of the ARB on leukocyte adhesion in hypoxia-induced retinal neovascularization[17] and endotoxin-induced retinal inflammation.[18] Leukocyte adhesion to the retinal vasculature is a critical antecedent event to vascular hyperpermeability,[3] obliteration,[4] and proliferation,[2] all of which represent the pathogenesis of diabetic retinopathy. The present data indicated AT1-R blockade as a possible therapeutic strategy for preventing leukocyte adhesion and subsequent microvascular complications in diabetic retinopathy. Similarly, acridine orange leukocyte fluorography, capable of evaluating extravasated leukocytes in the retinal tissue,[39] revealed the suppres-

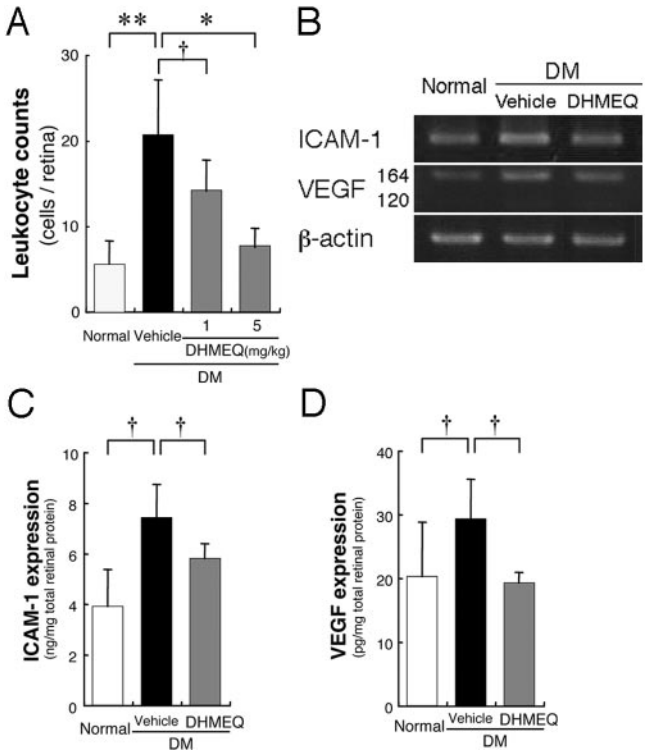

FIGURE 4. Inhibitory effects of NF-κB blockade on retinal adherent leukocytes (**A**) and retinal expression (**B**) and production (**C**, **D**) of inflammatory molecules. Treatment with DHMEQ significantly decreased adherent leukocytes (**A**). The mRNA expression (**B**) and protein levels (**C**, **D**) of ICAM-1 and VEGF were significantly suppressed by NF-κB blockade with DHMEQ (5 mg/kg). ($n = 8$; $^{**}P < 0.001$, $^*P < 0.01$, $\dagger P < 0.05$).

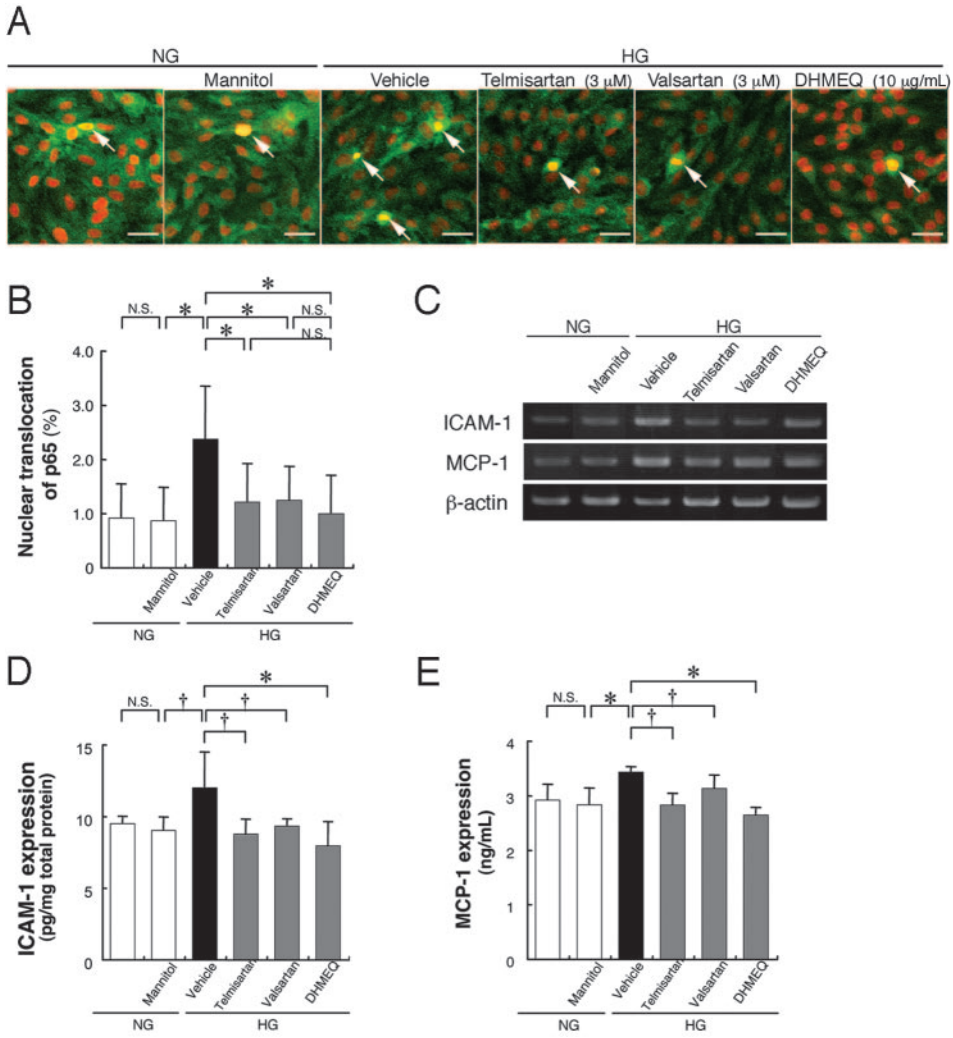

FIGURE 5. Nuclear translocation of NF-κB p65 (*arrows*), enhanced by high glucose, was suppressed by telmisartan or valsartan to levels similar to those with DHMEQ (**A**). Nuclear NF-κB p65 localization was visualized in the superimposed image showing *yellow* nuclei arising from the *green* fluorescence of the anti-NF-κB p65 antibody and *red* fluorescence from nuclei with TOTO-3. Scale bar, 40 μm. (**B**) Average ratio of the number of cells with nuclear p65 staining to the number of total cells per microscopic field. In vitro effects of ARBs or DHMEQ on mRNA expression (**C**) and protein levels (**D**, **E**) of ICAM-1 and MCP-1 in b-End3 cells. The mRNA expression and protein levels of ICAM-1 and MCP-1, elevated by high glucose, were significantly suppressed by telmisartan, valsartan, or DHMEQ. ($n = 8$; $^*P < 0.01$, $\dagger P < 0.05$).

sive effect of AT1-R blockade on leukocyte transmigration in the diabetic retina,[40] supporting our current data on leukocyte adhesion to the vessel walls (Fig. 2).

The present in vivo analyses demonstrated the involvement of VEGF and ICAM-1 as molecules responsible for the ARB-induced suppression of leukocyte adhesion in the diabetic retina (Fig. 3). ICAM-1, constitutively expressed on vascular endothelial cells at a low level, is swiftly upregulated during inflammation, resulting in enhancement of leukocyte–endothelial cell interaction.[41] Previous studies of donor eyes from diabetic subjects[12] and experimentally induced diabetes[5,7] demonstrated that retinal ICAM-1 expression was elevated together with leukocyte adhesion and infiltration. Antibody-based blockade or genetic ablation of ICAM-1 led to significant suppression of vascular hyperpermeability in early diabetes[5] or capillary loss in established diabetes.[6] VEGF, a potent angiogenic and proinflammatory factor, plays a central role in the pathogenesis of diabetic retinopathy. In patients with diabetic retinopathy, VEGF levels in the intraocular fluid were increased not only during the proliferative stage,[42,43] but also during the nonproliferative stage.[44] VEGF is also known as the upstream stimulant for ICAM-1 expression in diabetes.[3,7] Reasonably, anti-VEGF agents have been applied to eyes with diabetic macular edema in recent clinical trials.[45] Angiotensin II levels are elevated and correlated with VEGF levels in the vitreous fluid of patients with diabetic macular edema.[37] It has been shown to induce ICAM-1[46] and VEGF[47] via AT1-R in previous in vivo and in vitro studies, supporting the present data on the ARB-induced suppression of these inflammation-related molecules in the diabetic retina (Fig. 3). In contrast to AT1-R blockade, AT2-R blockade in our present study did not alter retinal expression of VEGF and ICAM-1. In the rodent model of oxygen-induced retinopathy, AT2-R blockade with PD123319 led to significant suppression of VEGF.[31] This divergence may be attributable to the difference in stimuli for VEGF induction (i.e., hyperglycemia versus hypoxia/ischemia). Long-term administration of PD123319 with the duration of 4 weeks (1 week in the present study) led to significant suppression of VEGF in the diabetic retina.[26] AT2-R is suggested to play a more chronic role in the pathogenesis of diabetic retinopathy than does the AT1-R/NF-κB pathway, which causes acute retinal inflammation.[18,48] So far, no data have been presented concerning AT2-R's blocking effect on retinal ICAM-1. The present finding that AT2-R blockade did not affect retinal ICAM-1 is compatible with its negligible effect on retinal leukocyte adhesion (Fig. 2).

Since NF-κB is suggested to induce the expression of various inflammatory molecules as a downstream pathway via AT1-R,[21] we investigated the proinflammatory role of NF-κB in the diabetic retina. Of note, inhibition of NF-κB nuclear translocation led to significant suppression of cellular and molecular inflammation-related parameters, including the number of adherent leukocytes and the expression of VEGF and ICAM-1 in the diabetic retina (Fig. 4), to the similar levels observed in the

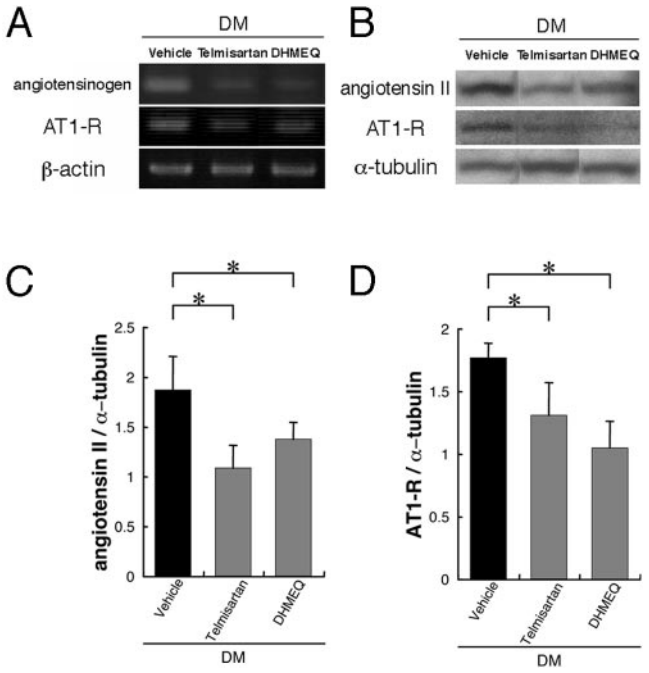

FIGURE 6. Suppression of the diabetes-induced upregulation of angiotensin II and AT1-R by AT1-R or NF-κB inhibition. RT-PCR (**A**) and Western blot (**B–D**) for expression and production of angiotensin II and AT1-R in the retina. (**A**) Retinal mRNA expression of angiotensinogen and AT1-R was substantially suppressed by AT1-R blockade with telmisartan (5 mg/kg) or NF-κB inhibition with DHMEQ (5 mg/kg). (**B–D**) Similarly, telmisartan or DHMEQ significantly suppressed retinal protein levels of angiotensin II and AT1-R. Data are the ratio of angiotensin II (**C**) and AT1-R (**D**) to α-tubulin. ($n = 6$; $^*P < 0.01$).

AT1-R-blocking data (Fig. 3). NF-κB is typically a heterodimer that consists of the p65 and p50 proteins. In the cytoplasm, NF-κB is usually inactive without stimulation because of its binding of I-κB, which prevents nuclear translocation of NF-κB. After I-κB phosphorylation and degradation due to various stimuli, NF-κB, capable of entering the nucleus and binding the κB sequence, promotes the transcription of target genes including VEGF, ICAM-1, and MCP-1.[20] Unlike other NF-κB inhibitors that block I-κB phosphorylation, DHMEQ used in the present study is unique in terms of acting at the level of the nuclear translocation of NF-κB.[27] Recent studies have established the inhibitory effects of DHMEQ on in vitro cytokine expression[49] and in vivo tumor growth and angiogenesis.[50] The present data on the role of NF-κB in diabetes-induced retinal inflammation (Fig. 4) are compatible with previous studies showing that NF-κB inhibition with pyrrolidine dithiocarbamate led to significant suppression of ischemia-induced retinal neovascularization[51] and endotoxin-induced ocular inflammation,[48] both of which recently have been shown to be mediated by AT1-R.[17,18]

Hyperglycemia is a primary factor that causes diabetic microangiopathy by enhancing leukocyte–endothelial cell interaction.[52,53] To confirm the molecular mechanisms involving the ARB-induced anti-inflammatory effects on the diabetic retina, the in vitro culture system with microvascular endothelial cells stimulated with high glucose was used. AT1-R blockade led to significant suppression of glucose-induced NF-κB nuclear translocation to the similar level of the NF-κB inhibitor DHMEQ application (Figs. 5A, 5B). NF-κB activation was shown to be involved in glucose-induced upregulation of ICAM-1[53] and MCP-1[54] in endothelial cells. Our in vitro data showed that the ARB-induced suppressive effects on glucose-induced upregulation of ICAM-1 and MCP-1 expression (Figs. 5C, 5D) were mediated by the inhibition of the NF-κB pathway. MCP-1 is a member of the C-C chemokine family known as a potent chemoattractant for monocytes and macrophages. Recent clinical data demonstrated that MCP-1 was increased in the vitreous fluid[11] and the fibrovascular tissue[55] in eyes with proliferative diabetic retinopathy, suggesting that MCP-1 is implicated in the pathogenesis of diabetic retinopathy. In the present in vitro experiments (Fig. 5), we used vascular endothelial cells derived from the brain. Although the diabetic brain and the retina show several inflammatory changes including the level of ICAM-1 expression[56] and leukocyte adhesion,[57] the diabetic brain does not develop severe vascular abnormalities as seen in diabetic retinopathy, suggesting a limited interpretation of our in vitro data in brain endothelial cells used to explain the pathogenesis of diabetic retinopathy.

In addition to its suppressive effect on the inflammatory parameters, ARB or DHMEQ treatment attenuated diabetes-induced enhancement of retinal angiotensin II and AT1-R production per se (Fig. 6). During the tissue RAS activation, angiotensin II was reported to stimulate further production of angiotensinogen via the AT1-R/NF-κB pathway, showing a positive feedback loop or vicious cycle.[58] AT-1R expression has also been shown to be regulated by NF-κB.[59] The present data on the suppressive effect of ARB or DHMEQ treatment on the pathogenic ligand-receptor system per se indicates the existence of the RAS/NF-κB-mediated vicious cycle in the diabetic retina. This is compatible with our recent report showing the suppression of AT1-R by blocking AT1-R in endotoxin-induced retinal inflammation.[29]

Collectively, the present in vivo and in vitro findings suggest the AT1-R/NF-κB pathway as a therapeutic target to prevent the development of diabetic retinopathy. The use of two different ARBs throughout the current experiments confirmed that the ARB-induced suppression of diabetes-induced retinal inflammation is a class effect. In clinical practice, ARBs are safely and widely used in patients with hypertension. A randomized controlled trial indicated that tight blood pressure control with a β-blocker or an ACE inhibitor prevents the progression of diabetic retinopathy and deterioration in vision.[60] Reasonably, AT1-R blockade is suggested not only to suppress diabetes-induced retinal inflammation but also to improve the systemic background contributing to diabetic retinopathy. The present study is likely to provide a molecular basis for the results in a clinical study that showed that the RAS blockade with an ACE inhibitor results in significant suppression of the progression of diabetic retinopathy.[23] It is notable, however, that there are indeed a large number of normotensive patients with diabetic retinopathy who have the potential risk of hypotension caused by antihypertensive agents. NF-κB, a downstream mediator of AT1-R without affecting systemic blood pressure (Table 2), is therefore considered to be an alternative and novel therapeutic target to control several inflammatory molecules related to diabetic retinopathy.

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