

Effect of angiotensin II type 1 receptor blocker and angiotensin converting enzyme inhibitor on the intraocular growth factors and their receptors in streptozotocin-induced diabetic rats

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Abstract

• **AIM:** To investigate the effect of angiotensin II type 1 receptor blocker (ARB) and angiotensin converting enzyme inhibitor (ACEI) on intraocular growth factors and their receptors in streptozotocin-induced diabetic rats.

• **METHODS:** Forty Sprague-Dawley rats were divided into 4 groups: control, diabetes mellitus (DM), candesartan-treated DM, and enalapril-treated DM (each group, $n=10$). After the induction of DM by streptozotocin, candesartan [ARB, 5 mg/(kg · d)] and enalapril [ACEI, 10 mg/(kg · d)] were administered to rats orally for 4wk. Vascular endothelial growth factor (VEGF) and angiotensin II (Ang II) concentrations in the vitreous were measured using enzyme-linked immunosorbent assays, and VEGF receptor 2 and angiotensin II type 1 receptor (AT1R) levels were assessed at week 4 by Western blotting.

• **RESULTS:** Vitreous Ang II levels were significantly higher in the DM group and candesartan-treated DM group than in the control ($P=0.04$ and 0.005 , respectively). Vitreous AT1R increased significantly in DM compared to the other three groups ($P<0.007$). Candesartan-treated DM rats showed higher vitreal AT1R concentration than the enalapril-treated DM group and control ($P<0.001$ and $P=0.005$, respectively). No difference in vitreous Ang II and AT1R concentration was found between the enalapril-treated DM group and control. VEGF and its receptor were below the minimum detection limit in all 4 groups.

• **CONCLUSION:** Increased Ang II and AT1R in the hyperglycemic state indicate activated the intraocular renin-angiotensin system, which is inhibited more effectively by systemic ACEI than systemic ARB.

• **KEYWORDS:** angiotensin converting enzyme inhibitor; angiotensin II type 1 receptor blocker; diabetic rat; intraocular renin-angiotensin system

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INTRODUCTION

The renin-angiotensin system (RAS) is known to play an important role in physiologic and pathologic conditions of the systemic vascular system. Angiotensin II (Ang II) is a major effector molecule that regulates various growth factors in the RAS^[1-4]. Independent of the systemic RAS, local RASs have been observed in several organs including the eye^[5-9]. Intraocular RAS is associated with intraocular pressure, ocular blood flow, and the production of vascular endothelial growth factor (VEGF)^[8-11].

Previous studies suggest that intraocular RAS has pathogenic roles on vascular proliferation and macular edema in diabetic retinopathy (DR)^[12-13]. Elevation of intraocular Ang II promotes VEGF and vascular endothelial growth factor receptor 2 (VEGFR-2) by stimulating angiotensin II type 1 receptor (AT1R) and induces vascular permeability and retinal neovascularization in the pathologic condition^[1,6,13-15]. On the basis of these findings, RAS inhibition has been highlighted to treat and prevent DR. To inhibit the RAS pathway, two major therapeutic agents, angiotensin II type 1 receptor blocker (ARB) and angiotensin converting enzyme inhibitor (ACEI), have been widely used. Clinical and experimental studies showed that treatment with ARB and ACEI had a beneficial effect on retinopathy progression in diabetes mellitus (DM) patients^[15-20], and improvements were seen in

blood retinal barrier breakdown, hyperpermeability, and retinal neovascularization in DR rats^[21-23]. However, inhibiting the RAS using systemic ARB and ACEI have not shown consistent outcomes for DR^[24-26].

Focusing on the effects of systemic ARB and ACEI on the intraocular RAS pathway and their pathologic receptors, we investigated the concentrations of Ang II, VEGF, AT1R, and VEGFR-2 in the vitreous after oral administration of candesartan (ARB) and enalapril (ACEI) in diabetic rats.

MATERIALS AND METHODS

Animal Preparation Forty inbred male Sprague-Dawley (SD) rats, weighing 200-250 g at 6wk of age, were purchased from Koatech Inc (Pyeongtaek, Korea). The rats were housed in standard 12h dark-light cycles at room temperature of approximately 23 °C and permitted free access to deionized water and standard rat chow for 7d after their arrival. The rats were divided to 4 groups (10 rats per group): control, DM, candesartan-treated DM, and enalapril-treated DM.

Care, use, and treatment of all animals in this study were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the institutional guidelines of Pusan National University. This study was approved by the Institutional Animal Care and Use Committee (PNU-IACUC, approval number: PNU-2013-0373) of Pusan National University.

Induction of Diabetes Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA; 60 mg/kg, in 10 mmol/L citrate buffer, pH 4.5). Rats in the control group received an equivalent amount of citrate buffer alone. Two days after STZ injection, we checked the rats' blood glucose levels using a blood glucose meter (New caresens N, I-Sens, Seoul, Korea) on the tips of their tails. Diabetes was defined as a blood glucose level >300 mg/dL 24h after STZ injection. Blood glucose levels were examined once per week to verify the maintenance of the diabetic condition throughout the study.

Oral Administration of Angiotensin II type 1 Receptor Blocker and Angiotensin Converting Enzyme Inhibitor for Diabetic Rats We administered candesartan [Atacand; Astrazeneca AB, Karlebyhus, Astraallén Södertälje, Sweden, 5 mg/(kg·d)] to the candesartan-treated DM group and enalapril [Lenipril; JW Pharmaceutical, Dangjin, South Korea, 10 mg/(kg·d)] to the enalapril-treated DM group orally *via* gastric sonde (BioGenomics, Seongnam, South Korea) for 4wk. Control and DM groups received the same volume of phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO, USA; 10 mL/kg) orally.

Preparation of Retina and Vitreous At week 4, we sacrificed the rats by CO₂ gas inhalation, followed by enucleation. The eyes were stored immediately at -80 °C. After the anterior segment was removed, the vitreous was meticulously

separated and transferred into sterile tubes. A full-thickness specimen including the retina and sclera was obtained at the same distance inferiorly from the optic disc. The tissues were prefixed with 2.5% glutaraldehyde (4 °C; phosphate buffer, pH 7.2) and were postfixated with 1% osmium tetroxide in the same buffer. The materials were dehydrated with a series of graded ethyl alcohol and were embedded in epoxy resin (Epon 812 mixture). Sections (1 μm) were stained with 1% toluidine blue for light microscopy. Thin sections were then examined with a light microscope (BX-50, Olympus, Japan).

Western Blot Analysis The separated vitreous were homogenized in protein extraction solution (PRO-PREP, iNtRON, Korea) and placed on ice for 30min. The supernatants were collected after centrifugation at 12 000 rpm for 10min at 4 °C. Protein concentrations were quantified using a Bicinchoninic acid Protein Assay Kit (BCA, Pierce, USA) according to the manufacturer's instructions. Extracted proteins were resolved on a 8% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, USA) by electroblotting. Nitrocellulose blots were blocked with 5% non-fat dry milk for 1h in Tris buffered saline with Tween-20 (TBS-T) buffer (20 mmol/L Tris pH 7.4, 137 mmol/L NaCl, and 0.1% Tween-20) at room temperature. Blots were incubated overnight at 4 °C with the appropriate primary antibody (VEGF Receptor 2, #2479, Cell Signaling, USA-dilution; 1:500) (AT1R, ADI-905-743, Enzo Life Science, USA-dilution; 1:300) (GAPDH, ab8245, Abcam, UK-dilution; 1:2000) in TBS-T buffer. Immunoreactive bands were detected using an anti-rabbit peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NY, USA) and visualized by enhanced chemiluminescence (ECL detection kit, Amersham Pharmacia Biotech, Piscataway, NY, USA). Protein expression was calculated by height, area, and optical density of the bands using a gel imaging system (UVIpro, UVItec Limited, UK).

Enzyme-linked Immunosorbent Assay for Vitreous The undiluted vitreous samples from frozen eyes were obtained and transferred into sterile tubes. The vitreous samples were immediately stored at -80 °C until use. The frozen biopsies were defrosted, homogenized in protein extraction solution (PRO-PREP, iNtRON, Korea), and placed on ice for 30min. The supernatants were collected after centrifugation at 12 000 rpm for 10min at 4 °C. The VEGF and Ang II protein levels in the vitreous were determined using the rat VEGF Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) and Ang II enzyme immunoassay kit (Spi Bio Bertin Pharma, Montigny le Bretonneux, France) according to the manufacturer's instructions. Optical density was read with a microplate reader (Emax, Molecular Devices, USA) at 450 nm or 405 nm (for VEGF and Ang II, respectively). The VEGF and Ang II concentrations (pg/mL) were calculated from a standard curve.

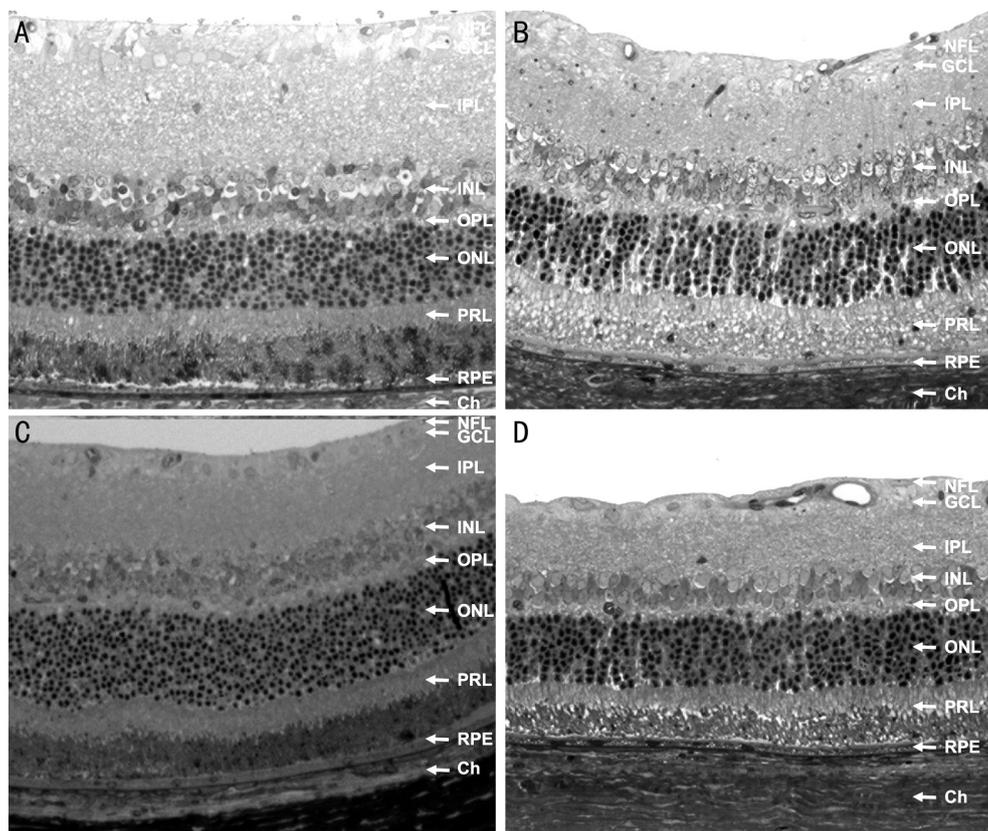


Figure 1 Histologic findings of retinal tissues The type of specimen: full thickness retina & choroid, original magnification: $\times 100$, and stain: 1% toluidine blue A: Controls; B: DM; C: Candesartan-treated DM; D: Enalapril treated DM groups. NFL; Nerve fiber layer; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; PRL: Photo receptor layer; RPE: Retinal pigment epithelium; Ch: Choroid.

The minimum detectable concentrations using the ELISA kits were 8.4 pg/mL for VEGF and 1.5 pg/mL for Ang II.

Statistical Analysis Statistical comparisons were performed with one way of analysis of variance (ANOVA), and the Kruskal-Wallis test. Statistical analysis was performed with the PASW statistics software (IBM SPSS software, New York, USA).

RESULTS

Blood Glucose Level, Body Weight, and Histology Blood glucose and body weight measured of the rats at week 4 are shown in Table 1. DM, candesartan-treated, and enalapril treated DM groups maintained high non-fasting blood glucose levels through week 4. Body weights were reduced in DM, candesartan-treated DM, and enalapril-treated DM groups compared to baseline ($P < 0.05$), but the control group gained body weight through week 4.

Histology showed no apparent abnormal findings in the retinal layers of all four groups (Figure 1).

Levels of Angiotensin II and Vascular Endothelial Growth Factor in Vitreous Table 2 presents vitreous Ang II concentration in the control, the DM group, the candesartan-treated DM group, and the enalapril-treated DM group. Vitreous Ang II levels were significantly higher in DM and candesartan-treated DM groups than in the control group ($P = 0.04$ and

Table 1 Body weight change 4wk after induction of diabetes

Groups	Body weight (g)	
	Baseline	4wk
Control	334.50 \pm 13.82	378.33 \pm 67.50
DM	294.33 \pm 14.87	225.16 \pm 40.47
Candesartan-treated DM	310.83 \pm 8.54	264.00 \pm 21.84
Enalapril-treated DM	315.67 \pm 16.22	243.67 \pm 44.22

DM: Diabetes mellitus.

Table 2 Vitreous angiotension II levels at week 4

Groups	Angiotensin II level (pg/mL)	P
Control	26.89 \pm 9.15	
DM	42.45 \pm 9.39	0.04 ^a
Candesartan-treated DM	47.11 \pm 14.63	0.005 ^b
Enalapril-treated DM	36.09 \pm 12.35	0.15 ^c

DM: Diabetes mellitus. ^aMann-Whitney *U* test was conducted between control and DM group; ^bMann-Whitney *U* test was conducted between control and candesartan-treated DM group; ^cMann-Whitney *U* test was conducted between control and enalapril-treated DM group.

0.005, respectively), and there was no difference between the enalapril-treated DM group and control.

VEGF concentrations were below the minimum detection limit in all 4 groups.

Vitreous Concentrations of Angiotensin II Receptor Type 1 and Vascular Endothelial Growth Factor Receptor 2

Figure 2 shows AT1R expression by Western blot assay. The DM group had a significantly higher concentration of vitreous AT1R than candesartan-treated DM ($P<0.001$), enalapril-treated DM ($P=0.006$), and control groups ($P=0.001$). Concentration of AT1R was higher in the enalapril-treated DM group than the control ($P=0.005$) and the candesartan-treated DM group ($P<0.001$). AT1R expression was comparable between the enalapril-treated DM group and the control group. The concentration of VEGFR-2 was below the minimum detection limit in all 4 groups.

DISCUSSION

The present study showed that intraocular Ang II and AT1R significantly increased in rats with DM. These results agree with previous studies, showing that RAS in the plasma as well as eye were correlated positively with severity of DM or retinopathy^[12,14,27]. Systemic enalapril effectively inhibited the expression of Ang II and AT1R in the vitreous. Although candesartan also reduced the vitreous AT1R concentration, it was less effective than enalapril, and did not prevent elevation of Ang II in the vitreous of DM rat.

Among the various proteins associated with the RAS, such as AT1R, AT2R and Mas receptor, AT1R is known as a major pathologic receptor in the eye. Ang II secretion can be promoted by hyperglycemic and hypoxic conditions, and may cause reduction in retinal capillary blood flow, increases in retinal vascular hyperpermeability, and decreased neovascularization by activation of AT1R^[21,23-24,28]. It has been suggested that inhibiting the RAS will be beneficial in management of DR.

Both ACEI and ARB inhibit RAS, but at different steps. ACEI inhibits the conversion of Ang I into Ang II, and ARB blocks AT1R selectively. Regarding clinical trials for DR, DM patients on lisinopril, another ACEI, showed a 50% reduction in DR progression compared to placebo after adjusting glycemic status^[16]. Candesartan also reduced progression of DR in early stages^[17-18]. Both enalapril (ACEI) and losartan (ARB) reduced DR progression in type 1 DM patients^[19]. However, the effect of systemic RAS inhibition on the intraocular RAS is still unclear.

Comparison of systemic ACEI and ARB administration to inhibit intraocular RAS was performed by Moravski *et al*^[15]. They demonstrated that the ACEI lisinopril reduced VEGF and AT1R mRNA expression in retinal tissues of Ren2 rats, but the ARB losartan did not. However, this outcome might have resulted from the different characteristics or dosages of drugs, rather than the common pharmacological action of ACEI or ARB, because ARB telmisartan inhibited VEGF mRNA expression in bovine retinal pericytes^[29]. Our results demonstrated that systemic enalapril reduced the vitreous

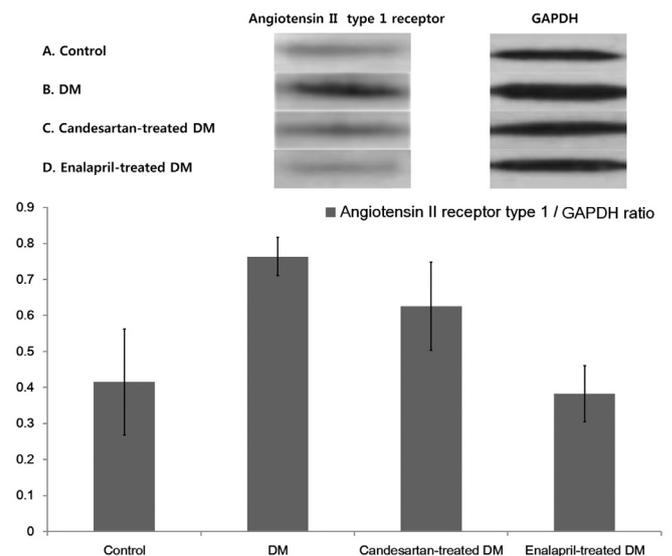


Figure 2 Western blotting for AT1R in vitreous.

Ang II and AT1R in DM rats to the level of control rats, but candesartan did not. In human studies, we previously reported that proliferative diabetic retinopathy (PDR) patients receiving various systemic ARBs did not have reduction of vitreous VEGF concentration^[25], but Hogeboom van Buggenum *et al*^[26] demonstrated that PDR patients receiving various ACEIs did show a decrease in vitreous VEGF expression. Accordingly, these findings imply that systemic ACEI may be more effective for inhibiting the intraocular RAS pathway than systemic ARB, which selectively blocks receptors.

VEGF and VEGFR also were reportedly elevated in intraocular tissues in diabetic conditions^[14,22-23,25-26,29], which is associated with activated AT1R by Ang II^[1,6,13-15]. But, we did not find VEGF and VEGFR-2 in either control or DM rats. These conflicting outcomes may result from different specimens used. Previous *in vivo* experimental studies showing high VEGF expression in diabetes measured VEGF and VEGFR concentration in retinas^[21-24], whereas we assessed the vitreous. An elevation of VEGF and VEGFR in retinal tissues may not be enough to spill over into the vitreous cavity in our study. In human studies, increased vitreous VEGF has been reported in PDR patients^[13-14,25-26], an advanced stage of DR. If the retina were in a more hypoxic condition such as in the oxygen induced retinopathy (OIR) model, an advanced DR model with new vessel formation, elevated VEGF in the vitreous may be detectable, although in previous studies VEGF in the vitreous was 10-fold lower than in the retina^[30-31].

There are some limitations to our study. First, it is based on an animal model and the study was designed to investigate short-term changes. The response to systemic ACEI and ARB may differ in rats compared to humans. Thus, our results from this experimental animal model may not reflect the clinical course in humans directly. Second, this study could lack sufficient power to detect the beneficial effect of ACEI and ARB, due

to the small number of animals used. A larger number of animals tested could generate a different outcome. Finally, the intraocular activity of systemic medication may vary according to formulas or doses, and the direct measurement of intraocular concentrations of drug and intraocular growth factors will provide further information regarding the efficacy of systemic ACEI and ARB.

In conclusion, vitreal Ang II and AT1R can increase in DM. In such a condition, systemic ACEI is likely to be more effective to inhibit the pathologic activation of intraocular AT1R than ARB.

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