Angiotensin II and Its Receptor Subtypes in the Human Retina

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PURPOSE. To quantify and evaluate the distribution of angiotensin II (Ang II) and its receptors in the human retina.

METHODS. Donor eyes were obtained within 12 hours postmortem and classified as hypertensive or normotensive and diabetic or nondiabetic, based on the donors' medical histories. Ang II in retina and vitreous was quantified by RIA. Ang II receptors were characterized and quantified by competitive membrane-binding assays. Ang II, its heptapeptide metabolite Ang(1-7), and AT1 and AT2 receptors were localized by immunohistochemistry and confocal imaging.

RESULTS. Levels of Ang II in the retina were significantly higher than in vitreous (P < 0.05). Ang II in the diabetic retina had a higher median compared with that in the nondiabetic retina. Ang II and Ang(1-7) colocalized in retinal Müller cells. The retina had the highest levels of Ang II receptors that were significantly higher than the optic nerve, retinal pigment epithelium–choroid complex, and ciliary body–iris complex (P < 0.05). AT1 receptors were more abundant than AT2 receptors in the retina. Immunoactivity for AT1 was detected in Müller cells and on blood vessels. AT2 receptors were localized throughout the Müller cells and nuclei of ganglion cells and neurons in the inner nuclear layer.

CONCLUSIONS. In the human retina, identification of Ang II and its bioactive metabolite Ang(1-7) in Müller cells suggests that these glial cells are able to produce and process Ang II. Ang receptors were localized in the blood vessels and neural cells. Local Ang II signaling may thus allow for autoregulation of neurvascular activity. Such an autonomous system could modulate the onset and severity of retinovascular disease. (Invest Ophthalmol Vis Sci. 2007;48:3301–3311) DOI:10.1167/iovs.06-1024

Diabetic retinopathy is one of the major complications of diabetes mellitus and the leading cause of visual loss and blindness in the adult population of the United States. It has been viewed as a disorder of the retinal vasculature1–2; however, evidence from numerous reports indicate that neural function of the retina is compromised before the vascular lesions are clinically diagnosed. At the cellular level, diabetes alters the structure and function of most cell types.3–9 Many factors have been implicated in the pathogenesis of diabetic retinopathy. Clinical and experimental studies have shown that the renin–angiotensin system (RAS) plays a pivotal role in the progression of the disease, presumably through local changes in blood flow and production of vascular endothelial growth factor (VEGF).10–22 Furthermore, Ang II may act as an inflammatory agent by enhancing vascular permeability through prostaglandins and VEGF and contribute to the recruitment of inflammatory cells by inducing chemokines and adhesion molecules.23,24 Although an independent RAS has not been established in the retina, many reports support the concept of a paracrine RAS in this organ.25–28 In the classic pathway, Ang II is produced by the sequential processing of plasma angiotensinogen by renal renin, followed by angiotensin converting enzyme (ACE) in the vasculature and the lungs (Fig. 1). However, in the past two decades, several laboratories32–36 have provided evidence of an expanded concept of the classic RAS to include independent Ang II-generating systems in the brain,37 heart,38 kidney,39,40 and adrenal glands.41,42 In the context of the neural retina, the localization of Ang II in neurons in many brain regions43–46 and in glial cells of the brain47 and the neuromodulatory actions of Ang II on central nervous system neurons is of interest.48,49 The complexity of the RAS was further expanded in 2000 by the discovery of ACE2,50 a “homologue” of ACE capable of producing Ang peptides such as angiotensin (Ang)-(1-7). Current research demonstrates that Ang-(1-7) may be the endogenous antagonist of the pathologic actions of Ang II.51,52 Most studies focused on the actions of the pharmacological agents that interrupt the RAS, but not on the effector peptide Ang II or its receptors.17,53–55 In humans, one study has reported the mRNA for angiotensinogen, renin, and ACE2 and only one study has shown the localization of Ang II, AT1 receptor, and ACE in retina.56 Hence, the purpose of the present study was to quantify and evaluate the distribution of Ang II and its receptors in retinal tissue. In this study, we extend the current knowledge on the retinal RAS by quantifying Ang II and its receptors and demonstrating the presence of Ang II, Ang-(1-7), and ACE2, as well as AT1 and AT2 receptors in the retina. In the retina, the Müller cells may be an important cellular source of Ang II, Ang-(1-7), and AT1 and AT2 receptors.

MATERIALS AND METHODS

Preparation of Donor Eye Tissue

Eyes were obtained from the Cleveland Eye Bank, in compliance with the Declaration of Helsinki, within 12 hours postmortem, and were dissected on a chilled tray. The eyes were cut at the ora serrata, and the anterior segment was lifted off. The vitreous body was isolated by gently shaking it out of the eye cup. The neural retina was carefully...
dissected from the retinal pigment epithelium (RPE) and isolated by cutting it at the optic nerve. In a second batch of six normotensive nondiabetic donors, the RPE–choroid complex, ciliary body–iris complex, and the optic nerve was also dissected for use in quantifying and characterizing Ang receptors. For biochemical analysis, the tissues were stored at $-80\,^\circ C$. For immunohistochemistry, the eyes were fixed in 4% paraformaldehyde-PBS. Donors for the quantitative estimation of Ang II levels were classified as hypertensive or normotensive and diabetic or nondiabetic according to the medical history from the Eye Bank. The diabetic donors included 8 females (age range, 56–86 years) and 9 males (49–85 years). The nondiabetic donors included 8 females (age range, 40–83 years) and 11 males (22–85 years). The characteristics of the donors are given in Table 1.

### Quantitation of Ang II in Retina and Vitreous

Frozen retina and vitreous samples were rapidly weighed and homogenized in chilled 75% ethanol containing 0.18% HCl and a mixture of protease inhibitors. Retinal and vitreous extracts were concentrated on C18 columns (Sep-Pak columns; Waters Associates, Milford, MA), evaporated to dryness, and reconstituted in 10 mM NaCl, 0.05% acetic acid, and 0.1% BSA. The levels of Ang II were quantified by RIA.

### Quantitation and Characterization of Ang II Receptor Subtypes

Ang II receptors were analyzed in three separate pools of four eyes from two donors per pool. To prepare the membranes, we homogenized tissues in a 10-fold volume (wt/vol) of chilled Hanks’ balanced salt solution containing protease inhibitors. The homogenates were centrifuged for 10 minutes at 1,200 g, and the resultant supernatant solution was centrifuged at $-80\,^\circ C$. Protein concentration was determined by the Bradford method. The Ang II receptors were quantified and characterized by competitive membrane binding assay.

#### Classical pathway of the RAS

- **Angiotensinogen**
- **Angiotensin I**
- **Angiotensin II**
- **Angiotensin III**
- **Angiotensin IV**

#### Alternate pathways of the RAS

- **Angiotensin-(1–5)**
- **Angiotensin-(1–7)**
- **Angiotensin-(1–9)**

**Figure 1.** Proteolytic pathways for angiotensin peptides. Ang II may be formed by the sequential enzymatic activity of renin and ACE or by proteases such as tonin, chymase, and cathepsins. In ocular tissues, in addition to ACE chymase, cathepsin B and D enzymatic activity has been detected.
**Immunocytochemistry: Ang II**

Immunolocalization of Ang II and Ang(1-7) was determined in frozen sections. Three nondiabetic donor eyes were evaluated. The following antibodies were used: rabbit polyclonal antibodies for Ang II (H-200-12, 1:200; Phoenix Pharmaceuticals, Belmont CA) and an in-house antibody for Ang(1-7) (CCF-Core 1, 1:20034,36). The sections were digested with pronase in acetate buffer (calcium 5 mM and sodium 10 mM [pH 7.5]) for 15 minutes at 37°C and washed with PBS. The sections were blocked with 2.5% BSA and incubated with the primary antibodies anti-Ang II and anti-Ang-(1-7). After an overnight incubation, the sections were blocked with 2.5% BSA and incubated with the primary antibodies: rabbit polyclonal antibodies for Ang II (H-200-12, 1:200; Phoenix Pharmaceuticals, Belmont CA) and an in-house antibody for Ang-(1-7) (CCF-Core 1, 1:20034,36). The sections were incubated with the primary antibodies with an equivalent dilution of nonimmune IgGs. Slides were mounted in antifade medium containing DAPI for labeling nuclei (Vectashield; Vector Laboratories, Inc.). Images were taken with a confocal laser-scanning microscope (Leica, Heidelberg, Germany).

**Ang Receptors**

Ang receptors were evaluated in three nondiabetic donors. The localization of the AT1 and AT2 receptors was determined in frozen sections with a monoclonal antibody to AT1 (ab99391, 1:20; Abcam, Cambridge, MA) and rabbit anti-human polyclonal AT2 antibody (1:100; Alpha Diagnostic International, San Antonio, TX). The colocalization of the AT2 receptor was evaluated with monoclonal antibodies against (1) glial fibrillary acidic protein (anti-GFAP, 1:200; Chemicon International, Inc., Temecula, CA), (2) glutamine synthetase (1:100; BD Biosciences, Franklin Lakes, NJ), (3) CD31 (M 0823, 1:100; DakoCytomation, Carpinteria, CA), (4) synaptophysin (S 5768, 1:100; Sigma-Aldrich, St. Louis, MO). The antibody binding was resolved with goat anti-rabbit and goat anti-mouse secondary antibodies coupled to Alexa Fluor 488 and 594, respectively. Specificity of the immunostaining was verified by substituting the primary antibodies with an equivalent dilution of nonimmune IgGs. Slides were mounted in antifade medium containing DAPI for labeling nuclei (Vectashield; Vector Laboratories, Inc.). Images were taken with a confocal laser-scanning microscope (Leica, Heidelberg, Germany).

**ACE2 Western Blot Analysis**

Four human donor retinas (three nondiabetic and one diabetic) were dissected as described earlier. The dissected tissue was quickly rinsed in 1× PBS and placed in chilled 0.5 M extraction buffer consisting of 0.5 M Tris-HCl (pH 8.0), 0.2 M NaCl, and 1% Triton X-100, with the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA as protease inhibitors. The protein concentration was determined by a BCA kit (Micro BCA Assay Reagent Kit; Pierce Chemical, Rockford, IL). For Western blot analysis, 150 μg of total protein was loaded per lane of retina and mouse kidney, along with a protein

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**Table 1. Characteristics of Human Donor Eyes**

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Det, death to enucleation; PMT, postmortem time (death to fixation); A, age; R, race; B, African American; W, white; S, sex; M, male; F, female; HTN, hypertension; MI, myocardial infarction; AC, acute cardiac events; CHF, chronic heart failure; *, no record; ACEI, angiotensin-converting enzyme inhibitor therapy; ARB, angiotensin II receptor blocker therapy.
marker (BMA ProSieve Color Protein Markers; BioWhittaker Molecular Applications, Rockland, ME) on a 7.5% SDS-polyacrylamide minigel. The gel was run at 100 V for 1.5 hours at room temperature. Separated tissue samples were electroblotted onto membranes (Immobilon-P, with a Semi-Dry Electrophoretic Transfer Cell; Bio-Rad, Hercules, CA) for 20 minutes at 18 V. After transfer, the membranes were blocked with 2% BSA/PBS for 1 hour, and the gel was stained (Gelcode Blue; Pierce Chemicals) to confirm equal loading of total protein.59 The membrane was then incubated in a diluted primary antibody, ACE2(1:1000 polyclonal anti-human ACE2 ectodomain antibody, raised against ACE2 residues; aa 18-740; AF933; R&D Systems, Minneapolis, MN) in 1% BSA/PBS at 4°C overnight. The membrane was washed three times using PBS/Tween 0.05% (5 minutes × 15 mL) and further incubated with the secondary antibody horseradish peroxidase anti-goat (1:5000; Vector Laboratories) in PBS/Tween 0.05% for 1 hour at room temperature. The membranes were washed three times as just described, and antibody detection was performed with enhanced chemiluminescence (ECL and SuperSignal West Pico Chemiluminescent Substrate; Pierce Chemical). Both gel and image were digitized on a densitometer (Quantity one Software and model 710 densitometer Bio-Rad). We also performed a specificity control for the ACE2-specific antibody by Western blot analysis, with and without preincubation of the antibody with the synthetic peptide used to generate the antibody.

Statistical Analysis

Summaries of quantitative data are expressed as the mean ± SEM with median and ranges reported, as appropriate. Group comparisons were performed with analysis of variance (ANOVA) followed by pair-wise comparison by the Fisher least significant difference test or by Wilcoxon rank sum tests. The association between quantitative variables was assessed for significant Spearman correlation. The criterion for statistical significance is \( P < 0.05 \).

Results

Ang II Peptide Levels

Ang II levels were determined in vitreous and retina of nondiabetic (\( n = 19 \)) and diabetic (\( n = 17 \)) donors. The levels of Ang II in vitreous was similar in both groups and ranged from 1 to 32 pg/mL (median, 9 pg/mL) in the nondiabetic and from 1 to 30 pg/mL (median, 6 pg/mL) in the diabetic group (Fig. 2A). These results are consistent with the reported levels.18,25 Hypertension had no influence on either group. Ang II levels in retinas of both groups had a wider range and higher median compared with the vitreous (Fig. 2B), ranging from 1 to 329 pg/mL (median, 28 pg/mL) and 5 to 367 pg/mL (median, 49 pg/mL) in nondiabetic and diabetic samples, respectively. The only levels of Ang II reported for retina are in rabbit.60 The levels in rabbit retina (273.9 ± 62.6 fmol/g; \( n = 6 \)) were significantly higher than in vitreous (3.9 ± 1 fmol/mL; \( n = 6 \)). This report clearly reveals the distinction between Ang II levels in retina and vitreous. Neither retinal nor vitreous Ang II demonstrated statistically significant association in cadaveric tissue. Data for hypertensive nondiabetic patients tended to cluster at the upper end of the range compared with results for hypertensive diabetic patients, who clustered at the lower end of the range. The immunoreactivity observed in this study may be an underestimate of the endogenous levels, because Ang II, like other neurotransmitters and neuromodulators, is rapidly metabolized, and only low levels are present within cells. Furthermore, Ang II may be more vulnerable to peptidase activity during the postmortem period resulting in weaker signal for Ang II. Plasma Ang II could not be determined for the donors, because we had no access to the donor blood samples. The normal range for human plasma Ang II determined in our laboratory, is 1.3 to 10.5 pg/mL (\( n = 25 \)).

Immunolocalization of Ang II

Figure 3 shows the localization of Ang II and Ang(1-7) in retinal sections of a normotensive nondiabetic donor. From the morphologic appearance, both Ang II and Ang(1-7) antisera labeled Müller cells. Immunoreactivity was marked in the Müller cell end feet for both peptides, in addition to the cellular processes spanning the retina. Ang(1-7) immunoreactivity in the cellular processes extended from the nerve fiber layer through the entire retina to the photoreceptor layer, whereas Ang II appears to extend up to the outer nuclear layer. Ang II and Ang(1-7) were also localized in the nerve fiber layer and the outer limiting membrane.

Ang II Receptors Levels

Ang II receptors were detected in all four tissues studied (Fig. 4). The receptor density was highest in the neural retina (12.1 ± 0.3 fmol/mg protein), was approximately half in the RPE-choroid complex (6.6 ± 1.1) and less in optic nerve (3.4 ± 0.1), and was lowest in ciliary body-iris complex (2.2 ± 0.4; Fig. 4A). In the ciliary body-iris, the receptors were exclusively AT1; however, in the other tissues, both AT1 and AT2 were present (Fig. 4B). In the retina, AT1 was predominant in the RPE–choroid complex; the percentage of AT1 was higher than
FIGURE 3. Localization of Ang II and Ang-(1-7) in the retina of a normotensive nondiabetic donor with cancer. (A) Ang II, (B) Ang-(1-7), and (C) negative control. Both Ang II and Ang-(1-7) were localized to the inner limiting membrane (yellow and red short open arrows), transverse fibers in the nerve fiber layer (yellow and red short solid arrows), Müller cell end feet (yellow and red open arrowheads), and Müller cell processes. Ang II in the Müller cell processes terminated at the top of the outer nuclear layer (yellow solid arrowhead), whereas Ang-(1-7) immunoreactivity spanned the retina up to the photoreceptor layer (red solid arrowhead). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar, 50 μm.

that of AT2. In the optic nerve, the percentages of AT1 and AT2 were comparable (Fig. 4B).

Immunolocalization of Ang II Receptors

Immunofluorescent staining for AT1 was detected in retinal blood vessels, Müller cell end feet, and photoreceptors (Fig. 5A1). Immunofluorescent staining for AT2 was detected around blood vessels, Müller cells, Müller cell processes, and nuclei of ganglion cells and neurons in the inner nuclear layer (Fig. 5A2). The AT2 receptor was colocalized with the AT1 receptor in Müller cell end feet (Fig. 5A3). The AT2 receptor was also colocalized with glutamine synthetase (Fig. 6B3) but not with GFAP (Fig. 6A3), CD31 (Fig. 6C3), or synaptophysin (Figs. 6D3-E3), markers for astrocytes, blood vessels, and synapses, respectively.

ACE2 Protein

The presence of ACE2 protein in the retinas from mouse kidney (Fig. 7A, lane 1), human nondiabetic (lanes 2–4) and diabetic (lane 5) retinas showed a single band at approximately 120 kDa. A control experiment performed after preabsorption of the antibody with the specific peptide antigen used to generate the antibody resulted in lack of staining, thereby verifying the specificity of the ACE2 detected in the retina (data not shown). ACE2 may undergo degradation during the period postmortem, resulting in a weaker signal for ACE2; thus, the immunoreactivity observed in this study may be an underestimation of the endogenous levels.

DISCUSSION

Detection of Ang II in the retina is consistent with the gene expression for angiotensinogen, renin, and ACE in human neural retina. Prorenin and renin are present in diabetic and nondiabetic vitreous. Intravitreous prorenin is increased in proliferative diabetic retinopathy (PDR) [Schiffman RM et al. IOVS 1992;33:ARVO Abstract 3346]. Ang I and Ang II are present in ocular fluids in diabetic and nondiabetic patients. ACE is present in human vitreous, and the levels are increased in PDR. Taken together, these findings provide strong support for a local RAS, with intraocular synthesis of Ang II, independent of the circulatory system in humans. Quantitative changes in Ang II and its receptors in the retina are fundamental elements that explain the mechanisms that underlie the benefits of ACE inhibitors and Ang II receptor blockers for the treatment of clinical and experimental ocular diabetic complications.

In diabetic patients, intraocular and serum levels of ACE, prorenin, and Ang II correlate with the severity of retinopathy. Plasma prorenin is increased with the onset of PDR in diabetic patients, and serum total renin (prorenin and renin) is two times higher in insulin-dependent diabetic patients than in normal subjects, suggesting that the RAS may be activated in patients with PDR. In diabetic patients with PDR, the prorenin concentrations in vitreous is 100 times the level expected on the basis of the plasma protein content of ocular fluid. Ang II levels are significantly higher in the vitreous of patients with PDR compared with nondiabetic persons, and/or diabetic patients without retinopathy. Activation of the intraocular RAS may lead to enhanced intraocular Ang II, which, in view of its effects on growth, may contribute to the development of PDR. Ang II is also a potent activator of TGF-β secretion, through which it stimulates matrix...
deposition and transactivates the VEGF receptor to mediate mitogenic stimulation of endothelial and epithelial cells.59,60
The role of the RAS extends beyond the regulation of blood pressure (BP). ACE inhibitors have effects far beyond simply reducing systemic vascular resistance.70,71 The inhibition of tension, is consistent with the premise that by blocking the RAS ACE inhibitors have beneficial effects, independent of their effects on BP.

Our data demonstrating a wide range in the levels of retinal Ang II within each clinical category may be a reflection of the heterogeneity of peptide expression and/or the accompanying therapeutic regimens needed to treat the multiple concurrent disease states; peptidase activity during the time postmortem may also play a role. The higher levels of Ang II in hypertensive nondiabetic patients compared with hypertensive diabetic patients may be due to the regular clinical management schedules resulting in improved compliance by the hypertensive patient with diabetes. The higher levels of Ang II in normotensive diabetic patients, compared with those with hypertension, could be due to the borderline and/or prehypertensive donors from the human finding with respect to ganglion cells, some similarities to those showing distribution in rabbit tissue, but differ in the human neural retina, localization of Ang II was evaluated in future studies to understand the molecular mechanisms of ACE inhibitors and Ang receptor antagonists that act on retinal targets. In patients receiving ACE inhibitor treatment, Ang(1-7) is elevated in the plasma72 and heart.73 ACE inhibition may have the following effects: (1) inhibit the metabolism of Ang I to Ang II by the classic pathway, whereas Ang II is synthesized by an alternate pathway(s). (2) ACE 2 is upregulated, and Ang II is hydrolyzed to Ang-(1-7) with high catalytic efficiency.74 (3) Ang I is also converted to Ang-(1-7) by neutral endopeptidase. (4) Ang-(1-7) catabolism to Ang-(1-5) is also inhibited. Thus, Ang-(1-7) is enhanced by multiple mechanisms under ACE inhibition. The physiological significance of Ang-(1-7) is currently under investigation. It raises the possibility that Ang-(1-7) may be the effector peptide producing the beneficial actions of alleviating experimental and clinical diabetic complications with ACE inhibition. Ang-(1-7) has vasodepressor actions51 and may have antiproliferative effects52 on vascular cells. Whether Ang-(1-7) is antiangiogenic or alters vascular activity in retina remains to be established.

Ang II and Ang-(1-7) are both localized in Müller cells, the principal glial cell of the retina. Ang-(1-7) is considered as the endogenous antagonist of the pathologic actions of Ang II; the present study has demonstrated that the two peptides are ideally located to serve this function efficiently. The localization of Ang in Müller cells is an important finding in view of the potential role for Ang II in the initial phase of the pathogenesis of diabetic retinopathy. In experimental diabetes, angiotensinogen is an acute-phase response protein that is upregulated in reactive retinal Müller cells.71 Ang II has been localized in rabbit retina69 but the level of immunoreactivity was low. In this study protease inhibitors were required to enhance Ang II immunoreactivity in Müller cells and Müller cell processes, amacrine cells, and along the external limiting membrane. This finding demonstrates the vulnerability of Ang II to peptidase activity. In the human neural retina, localization of Ang II was described in ganglion cells, cells of the inner nuclear layer, photoreceptor cells, and endothelial cells.50 Our data showing its distribution in the Müller cells and Müller cell processes are similar to those showing distribution in rabbit tissue, but differ from the human finding with respect to ganglion cells, some cells of the inner nuclear layer, and endothelial cells. The differences between these studies may be due to the differences in tissue integrity, because of postmortem time and tissue processing, resulting in altered sensitivity to the anti-Ang II antibody.

Ang II acts on two pharmacologically and structurally distinct receptors, AT1 and AT2. Both are seven-transmembrane-domain receptors.75 It is well established that activation of the AT1 receptor is responsible for growth promo-
tion, vasoconstriction, and hormone secretion. In contrast, the role of the AT2 receptor is less well established. It has been proposed that AT2 activation results in antiproliferation and vasodilation’s playing a critical role in antagonizing the actions of Ang II at the AT1 receptor. In addition to the classic receptors, Ang II may bind to acceptors on nuclear chromatin and initiate nuclear events that may result in protein synthesis and cell proliferation. Ang II localizes to the nucleus in several cell types, including neuronal cells.

ACE inhibitors and Ang receptor blockers may reduce the progression of diabetic retinopathy. Receptor antagonists
may be clinically superior because they target Ang II synthesized by both ACE-dependent and -independent pathways, whereas ACE inhibitors target only ACE-dependent Ang II formation. Our data reveal that Ang II receptors are present in ocular tissues, and retina has the highest levels. There are no reports of Ang II receptor quantitation in human ocular tissues. However, there are supporting data on receptor localization in human, rodent models, and tissue culture. In human donor retina, low-intensity AT1 immunoreactivity was detected around the ganglion cell layer, whereas in the present study we detected AT1 receptors in Müller cells and the blood vessels of the inner nuclear layer. The latter finding is consistent with the report in mouse retina and first demonstration of Ang binding sites in retinal vessels in feline retina. The localization of AT1 receptors in the retina blood vessels in transgenic Ren-2 rats, Sprague-Dawley rats, and rats with retinopathy of prematurity is consistent with our findings. The distribution of AT2 receptors in the inner nuclear layer and ganglion cell layer in the present study is consistent with the findings in rat; however, localization of the AT2 in human retina has not been reported previously. With respect to the localization of AT2 receptors also in the blood vessels reported for transgenic Ren-2 rats, Sprague-Dawley rats, and rats with retinopathy of prematurity, our data differ, in that we did not detect AT2 receptors in blood vessels.

The present study localized synaptophsin to the outer and inner plexiform layers, as reported by Kurihara et al. in mouse retina. However, our findings are different with respect to the colocalization of the AT2 receptor with synaptophsin observed by Kurihara et al. These differences may be due to a difference in species and/or detection protocols. The report of mRNA for both AT1 and AT2 in the human RPE cultures (Marin Catano ME et al. IOVS 2004;45:ARVO E-Abstract 1811) supports our observations in the RPE-choroid complex. AT1 and AT2 mRNA are also present in rodent optic nerve, giving credence to our findings. A unique feature of the present study is the nuclear localization of the AT2 receptor in the human ganglion cells and some neurons in the inner nuclear layer. There is evidence for polypeptide ligands and their membrane receptors to have signaling roles at the level of the nucleus. In the cerebellar cortex, using immunogold staining methods, Ang II was localized to nuclei of the cerebellar neurons. In isolated hepatocytes, Ang II was found to stabilize angiotensinogen mRNA. The discovery of the AT2 receptor in the nucleus may enable the function of Ang II in the nucleus to be elucidated.

A striking feature of Müller cells in human retina is that they contain all the components for the biosynthesis of Ang II: mRNA and protein for angiotensinogen, rennin, ACE, and ACE2 (Table 2). The discovery of Ang-(1-7) in the Müller cells is an important feature of this study, because Ang-(1-7) opposes the vasoconstrictor and proliferative actions of Ang II. ACE2, the enzyme that generates Ang-(1-7), is also present in the Müller cells, and its enzymatic activity is increased in diabetes. ACE2 activity may counterbalance the Ang promoting effects of ACE by preventing Ang II accumulation. Müller cell-specific regulation of Ang II and the interplay between Ang II and Ang-(1-7) may have important implications for normal and altered retinal physiology. Ang II in Müller cells is of special interest in view of its implication in enhancing vascular permeability and the role Müller cells play in the formation and maintenance of the blood-retinal barrier and regulation of blood flow. Moreover, neuronal impairment may occur much earlier in the pathogenesis of diabetic retinopathy than originally assumed. Although diabetic retinopathy has been viewed traditionally as a disorder of the retinal vasculature, recent evidence indicates that it also affects the glial and neural cells of the retina. The principal glial cell of the retina is the Müller cell, a specialized radial glial cell spanning the entire depth of the retina. Through its spatial arrangement, Müller cells intercalate between the vasculature and the neurons. Müller cells play an important role in the uptake of glucose from the circulation and its metabolism and transfer of energy to neurons. These functions are interdependent. Thus, when hyperglycemia prevails, Müller cell functions may be impaired. Ang II is a growth factor in vascular smooth muscle cells. AT1 receptors in the retina are well placed to target Müller cell Ang II. Ang II has powerful angiogenic actions and is associated with overexpression of angiogenic factors such as VEGF and Ang 2 that have an important role in the development of retinal neovascularization.

**Table 2. Distribution of the RAS Proteins and Peptides in the Human Neural Retina**

<table>
<thead>
<tr>
<th>RAS</th>
<th>Retinal Localization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensinogen</td>
<td>Müller cells</td>
<td>74</td>
</tr>
<tr>
<td>Ang I</td>
<td>Aqueous, vitreous, and subretinal fluid</td>
<td>26</td>
</tr>
<tr>
<td>Ang II</td>
<td>Müller cells, aqueous, vitreous, subretinal fluid, ganglion cells, retinal vessel endothelial cells, cells in the inner nuclear layer, and photoreceptor cells</td>
<td>*, 26, 56</td>
</tr>
<tr>
<td>Ang(1-7)</td>
<td>Müller cells</td>
<td>*</td>
</tr>
<tr>
<td>Renin</td>
<td>Müller cells and vitreous fluid</td>
<td>25, 27</td>
</tr>
<tr>
<td>ACE</td>
<td>Müller cells, ganglion cells, retinal vessel endothelial cells, cells in the inner nuclear layer, photoreceptor cells, and vitreous</td>
<td>56, 61, 81, 91</td>
</tr>
<tr>
<td>ACE2</td>
<td>Retina</td>
<td>*</td>
</tr>
<tr>
<td>AT1</td>
<td>Müller cells, retinal blood vessels, photoreceptors, and ganglion cells</td>
<td>*, 56</td>
</tr>
<tr>
<td>AT2</td>
<td>Müller Cells, nuclei of some inner nuclear layer neurons, and ganglion cell nuclei</td>
<td>*</td>
</tr>
</tbody>
</table>

Angiotensinogen, rennin, and ACE mRNA are also expressed in the human neural retina. Current study.
concentrations of prorenin in the vitreous leads to an blindness. In patients with PDR, it is conceivable that high and vitreous that eventually results in impaired vision and characterized by the ingrowth of new blood vessels into the retina promotes neovascularization. The biosynthesis of Ang II within activated local RAS, resulting in the generation of Ang II, which may have a role in the pathogenesis of diabetic retinopathy.

Although we appreciate that understanding the pathologic basis of disease is fundamental to the formulation of new treatments, we also realize that there are some limitations (age, advanced stages of disease, and the inability to investigate transduction pathways) inherent in these studies performed in donor tissues. Thus, disease models in which concurrent disease states and therapeutic regimens can be controlled are needed to identify the neurovascular mechanisms of Ang II that may have a role in the pathogenesis of diabetic retinopathy.

In conclusion, we have quantified for the first time Ang II and Ang receptors in human retina. This is a fundamental requirement for understanding the role of the RAS in diabetic retinal complications and therapeutic targeting of the ocular RAS. The significant features of this study are (1) the discovery of nuclear localization of the AT2 receptor in the ganglion cells and neurons in the inner nuclear layer; (2) the discovery of Ang-(1-7), the potential endogenous antagonist to vasopressor-growth promoter Ang II. We are presently working on models that will address the role of Ang II and Ang-(1-7) in normal, altered physiology and pathophysiology of the retina.

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