Angiotensin Levels in the Eye

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Purpose. Ocular tissues contain renin and ocular fluids contain prorenin in amounts that are too high to be explained by admixture with blood or diffusion from blood. It was the purpose of the present study to obtain further evidence for the presence of a local renin-angiotensin system (RAS) in the eye.

Methods. The authors measured the concentrations of angiotensins I and II (ANG I and II) in vitreous fluid and ocular tissues of anesthetized pigs and in human aqueous, vitreous, and subretinal fluid obtained during eye surgery.

Results. In tissues obtained from normal porcine eyes (anterior uveal tract, neural retina, retinal pigment epithelium + choroid), ANG I and II were 5- to 100-fold higher than could be accounted for by contamination with blood. ANG I and II in ocular tissues are therefore unlikely to be derived from the circulation. In porcine vitreous fluid, ANG I and II were close to the limit of detection. In addition, during a 2-hour infusion of ^{125}I-ANG I in the rabbit, ^{125}I-ANG I in vitreous fluid reached a level only 1% of the level in arterial plasma. Thus, in the presence of an intact blood-retinal barrier, little or no ANG I or II enters the vitreous compartment.

In human ocular fluids obtained from diseased eyes, ANG I and II levels were readily measurable and correlated linearly with the level of serum albumin, indicating that after partial breakdown of the BRB, diffusion of ANG I and II from the circulation into the eye may occur.

Conclusion. Results indicate that both ANG I and II are generated locally in ocular tissues with little leakage into ocular fluids. These findings, together with previously published data on renin and prorenin, show a high degree of compartmentalization of the RAS in the eye and are in agreement with similar findings in other tissues, where there is evidence for the existence of a local RAS. Invest Ophthalmol Vis Sci. 1994;35:1008–1018.

According to the classical concept, angiotensin I (ANG I) and angiotensin II (ANG II) are generated within the circulation by sequential cleavage of liver-derived renin substrate. Renin, synthesized in the kidney, cleaves this substrate to form ANG I. Angiotensin converting enzyme (ACE) converts ANG I to ANG II, a potent vasoconstrictor and a stimulant of the release of aldosterone from the adrenal. Recent evidence suggests that besides this circulating renin-angiotensin system, there also exist tissue or local renin-angiotensin systems—for instance in the adrenal, kidney, brain, testis, and ovary.[1–6] Local angiotensin production may therefore occur independently of the reaction of circulating renin with circulating renin substrate.

We detected prorenin, the inactive precursor of renin, in ocular fluids obtained from subjects with and without diabetes during eye surgery.[7] Prorenin concentrations were up to 100 times higher than was expected on the basis of the plasma protein content of the ocular fluid samples. In vitreous fluid from eyes affected by proliferative diabetic retinopathy, the prorenin concentrations were on average two times higher than in vitreous fluid from eyes of subjects without diabetes, also when allowance was made for the differences in plasma protein content. The renin levels in most ocular fluid samples were close to the detection limit of the assay.

Further studies in bovine eyes[8] showed indeed that both renin and prorenin were present in virtually all segments of the eye, in concentrations much too high to be explained by admixture with blood. The

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presence of ACE in the eye has been shown as well, not only in the retina, choroid, and ciliary body,[9] but also in aqueous fluid.[10] Moreover, using the polymerase chain reaction, we were able to show expression of the renin-, renin substrate-, and ACE-genes in human ocular tissue.[11] Taken together, these findings suggest that the eye has its own renin-angiotensin system (RAS) and that it is activated in eyes affected by proliferative diabetic retinopathy.

The function of an ocular RAS is not yet known. Its activation in the eyes of diabetic subjects with proliferative retinopathy may suggest that ANG II is involved in the development of neovascularization, as has been shown by others.[12,13] Because ANG II receptors have been detected in retinal blood vessels,[14] a role for the RAS in the regulation of retinal vascular tone is also conceivable.[15] Finally, the fact that renin inhibitors[16] and ACE inhibitors[17] lower intraocular pressure suggests that an intraocular RAS may play a role in aqueous humor dynamics.

To our knowledge, no measurements of angiotensins in the eye have been reported. In this study, we present data on angiotensin levels in porcine ocular tissues and fluids. To study whether plasma angiotensins reach the vitreous or aqueous fluid compartments, constant infusions of ^{125}I-ANG I were given to rabbits, and the levels of radiolabeled angiotensins in the eye were measured at various time intervals during these infusions. We also measured angiotensin levels in ocular fluid samples of subjects with and without diabetes. We further looked for the presence of ACE in these samples by measuring the amount of ^{125}I-ANG II formed after the addition of ^{125}I-ANG I.

SUBJECTS AND METHODS

Chemicals

[Ile5]-ANG-(1-10) decapeptide (ANG I), [Ile5]-ANG-(1-8) octapeptide (ANG II), and [Ile5]-ANG-(2-8) heptapeptide (ANG III) were obtained from Bachem (Bubendorf, Switzerland). [Ile5]-ANG-(2-10) nonapeptide (ANG-(2-10)) was obtained from Senn Chemicals (Dielsdorf, Switzerland). [Ile5]-ANG-(3-8) hexapeptide (ANG-(3-8)), [Ile5]-(4-8) pentapeptide (ANG-(4-8)), and [Ile5]-ANG-(1-7) heptapeptide (ANG-(1-7)) were obtained from Peninsula Laboratories (Belmont, CA). Methanol, ethanol, ortho-phosphoric acid (all analytical grade), trisodium citrate, and 1,10-phenantroline were obtained from Merck (Darmstadt, Germany). Bovine serum albumin was obtained from Sigma (St. Louis, MO). Disodium EDTA was obtained from Riedel de Haën (Scelze, Germany). Water for high-performance liquid chromatography (HPLC) was prepared with a Milli-Q system from Waters (Millford, MA). The renin inhibitor CGP 29,287 was a gift of Dr. K. Hofbauer (Ciba-Geigy, Basel, Switzerland). The renin inhibitor Ro 42,5892 was a gift of Dr. P. van Brummelen (Hoffmann-La Roche, Basel, Switzerland).

Preparation of Radiolabeled Angiotensins

Mono-iodinated ^{125}I-ANG I was prepared with the chloramine-T method and purified as described previously.[18,19] The specific radioactivity of the ^{125}I-ANG I preparation was approximately 3.6 * 10^6 counts per minute (cpm)/pmol (74 kBq/pmol). ^{125}I-labeled preparations of ANG II, ANG III, ANG-(3-8), ANG-(4-8), ANG-(2-10), ANG-(1-7), and tyrosine were also made.

Separation of Angiotensins by HPLC

Angiotensins and their metabolites were extracted from plasma, ocular fluid, or purified tissue homogenates by reversible adsorption to octadecylsilyl silica (SepPak C18, Waters, Millford, MA) and separated by reverse-phase HPLC, according to the method of Nussberger et al[20] with some modifications.[19] The SepPak cartridges were conditioned with 4 ml methanol and equilibrated by two washes with 4 ml of cold water. Samples were passed through the cartridge followed by two washes with 4 ml of cold water. Adsorbed angiotensins were eluted with 3 ml methanol into polypropylene tubes, and the methanol was evaporated under vacuum rotation, using a Savant Speed Vac concentrator (Savant Instruments, Farmingdale, NY). Separations were performed on a reversed-phase Nucleosil C18 steel column of 250 × 4.6 mm and 10 μm particle size. Mobile phase A was 0.085% ortho-phosphoric acid containing 0.02% sodium azide. Mobile phase B was methanol. The flow was 1.5 ml/min, and the working temperature was 45°C. Dried SepPak methanol extracts were dissolved in 100 μl of HPLC solvent (mobile phase A) and injected. Elution was performed as follows: 65% A–35% B from 0 to 9 minutes, followed by a linear gradient to 45% A–55% B until 18 minutes. The eluate was collected in 20-second fractions into polystyrene tubes coated with bovine serum albumin. The concentrations of ^{125}I-ANG I and its metabolites in the HPLC fractions were measured in the gamma counter. The fractions containing unlabeled ANG I and II were neutralized with 0.5 M sodium hydroxide and vacuum dried at 4°C.

Assay of Angiotensins

ANG I and ANG II concentrations were measured by radioimmunoassay after SepPak extraction and HPLC separation.[19] The ANG I antiserum crossreacted with ANG-(2-10) (100%) but not (less than 0.1%) with ANG II, ANG III, ANG-(3-8), ANG-(4-8), and ANG-(1-7). The ANG II antiserum crossreacted with ANG III (55%), ANG-(3-8) (73%), and ANG-(4-8) (100%), but not (less than 0.2%) with ANG I, ANG-(2-10), or

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ANG-(1-7). The lower limit of detection was 0.5 fmol/fraction for the ANG II assay, and 1.0 fmol/fraction for the ANG I assay.

Measurement of Albumin and Renin Substrate

Human albumin was measured by single radial immunodiffusion (LC and NOR-Partigen plates, Behringwerke, Marburg, Germany) according to the method of Mancini et al.[21] The concentration of renin substrate was determined as the maximum quantity of ANG I that was generated during incubation at 37°C and pH 7.5 with an excess of active purified human kidney renin in the presence of inhibitors of angiotensinases and ACE.[22] The lower limit of detection was 1 nmol/l.

Angiotensin Levels in Porcine Eyes

Eyes were taken from 20 anesthetized pigs (weight 25 to 50 kg, crossbred Yorkshire × Landrace, H.V.C., Hedel, The Netherlands). The pigs had been used for various pharmacologic studies not involving the eye. An adequate depth of anesthesia was maintained by the administration of 160 mg/kg alpha-chloralose (Merck, Darmstadt, Germany) via the superior caval vein, followed by continuous intravenous infusion of low-dose sodium pentobarbitone (5 mg/kg per hour). The eyes were dissected immediately after enucleation. Each eye was cut equatorially at the ora serrata, and the anterior segment was removed. The vitreous body was isolated by gently shaking it out of the eye cup. The neural retina was cautiously removed from the pigment epithelium with a thin glass rod and isolated by cutting it at the optic nerve. The choroid, with adhering retinal pigment epithelium layer, was isolated by dissecting it from the sclera with a pair of fine scissors. The anterior uveal tract, consisting of iris and ciliary body, was isolated by removing the lens from the anterior eye cup, then gently pulling the anterior uveal tract loose from the sclera and blotting it on dry paper to remove any adhering vitreous. Cornea, lens, and sclera were discarded, whereas neural retina, retinal pigment epithelium + choroid, anterior uveal tract, and vitreous were snap frozen on dry ice and stored at −70°C. The whole procedure took 2 to 3 minutes.

Simultaneously with the removal of the eyes, a peripheral blood sample was taken, after which the animals were killed. The blood (10 ml) was collected in a chilled polystyrene tube containing 0.5 ml inhibitor solution (containing 0.2 mM Ro 42,5892, 125 mM disodium EDTA, and 25 mM 1,10-phenantroline). The blood was centrifuged at 3000g for 10 minutes at 4°C. Plasma was stored at −70°C and assayed within 2 weeks as described above.

Pools of tissues obtained from 10 pigs (20 eyes) were homogenized with a Polytron PT10/35 (Kinematica, Luzerne, Switzerland) in an iced solution of 0.1 M HCl/80% ethanol according to the method of Chappell et al[23] with some modifications. In short, homogenates were centrifuged at 4°C for 20 minutes at 20,000g, and the supernatant was stored for 12 hours at −20°C. The supernatant obtained after a second centrifugation step (4°C, 20,000g, 20 minutes) was diluted 1:1 (vol/vol) with 0.1% ortho-phosphoric acid, stored for 4 to 6 hours at 4°C, and again centrifuged at 20,000g. The final supernatant was further diluted 1:1 with 0.02% ortho-phosphoric acid and concentrated on a SepPak column. Vitreous was concentrated on SepPak columns without further additions. SepPak extracts were applied to the HPLC as described above, and angiotensins were measured by RIA.

Breakdown of angiotensins during storage at −70°C was studied by adding ^{125}I-ANG I to frozen tissues 1 week before homogenization. The efficiency of the extraction was determined by the addition of 200,000 cpm ^{125}I-ANG I immediately before homogenization. Overall recovery was 59 ± 9% (mean ± SD, n = 6). Values were corrected for incomplete recovery.

^{125}I-ANG I Infusions in Rabbits

All procedures were in accord with the ARVO Resolution on the Use of Animals in Research. Rabbits were chosen for these experiments because they are small enough to obtain sufficiently high steady-state concentrations of ^{125}I-ANG I during infusion of relatively low amounts of radiolabeled ANG I, whereas at the same time they are large enough to allow the collection of several 1 to 2 ml blood samples during the infusion period. Three rabbits, weighing 2.9 to 3.3 kg, were anesthetized using intramuscular xylazine (Bayer, Leverkusen, Germany; 2 mg/kg) and ketamine HCl (Aesculaap BV, Boxtel, The Netherlands; 20 mg/kg). In addition, they received topical oxybuprocaine HCl (M.S.D., Haarlem, The Netherlands) for local anesthesia. The ACE inhibitor ramipril was injected intraperitoneally (1 mg/kg) shortly before the experiment. This was done to slow down the fast breakdown of infused ^{125}I-ANG I. ^{125}I-ANG I (mean concentration 3.4×10^6 cpm/ml) was infused at a rate of 0.1 ml per minute in the ear lobe vein during 2 hours. Arterial samples (1 to 2 ml) were taken from the carotid artery at 15, 30, 45, 60, 90, and 120 minutes after the start of the infusion. The blood was collected in prechilled tubes containing 0.1 ml of the above inhibitor solution and centrifuged at 3000g for 10 minutes at 4°C. One milliliter of plasma was counted in a gamma counter for measuring total radioactivity. After 1 hour, an aqueous and a vitreous sample were removed from one eye with an injection needle with a 25- and a 19-gauge respectively, and total radioactivity was counted in both samples. At the end of the infusion period, the animals were killed, and immediately aqueous and vitreous

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samples were taken from the second eye and counted in the gamma counter.

After the experiment, the ocular fluid and plasma samples were passed through SepPak columns and applied to the HPLC, as described before. Radiolabeled angiotensins were measured by gamma counting.

Human Studies

The research followed the tenets of the Declaration of Helsinki. Informed consent was obtained after the nature of the study had been explained.

Subjects Without Diabetes. Aqueous fluid was collected at the time of cataract extraction from 26 subjects (20 women, 6 men; mean age, 71 years; range, 38 to 92 years). Vitreous fluid aspirates were obtained from 18 subjects (11 women, 7 men; mean age, 57 years; range, 10 to 80 years). The samples were collected at the time of pars plana vitrectomy, which was performed because of recurrent retinal detachment due to proliferative vitreoretinopathy (n = 12), macula pucker (n = 3), or vitreous hemorrhage (n = 3). Subretinal fluid was obtained from 21 subjects (11 women, 10 men; mean age, 55 years; range, 13 to 79 years), with a rhegmatogenous (n = 20) or traumatic (n = 1) retinal detachment.

Subjects With Diabetes. Vitreous fluid was obtained from 24 subjects with diabetes (6 women, 18 men; mean age, 51 years; range, 21 to 75 years). The duration of diabetes ranged from 1 to 55 years. The eyes were affected by proliferative diabetic retinopathy, and vitrectomy was performed because of traction retinal detachment (n = 15) or vitreous hemorrhage (n = 9). Seventeen subjects were taking insulin. Neither subretinal nor aqueous fluid was collected from subjects with diabetes.

Collection of Plasma and Ocular Fluid Samples. Approximately 0.1 ml aqueous fluid was collected with a tuberculin syringe and a 27-gauge needle. The needle was introduced at the limbus of the cornea through the groove of the cataract incision. Vitreous fluid was aspirated before substitution fluid was infused into the vitreous. Subretinal fluid was aspirated transsclerally, after local diathermic coagulation of the choroid.

The ocular fluid samples were free of macroscopically visible blood and were collected in prechilled plastic tubes containing 25 μl of inhibitor solution (containing 2 μM CGP 29,287, 125 mM disodium EDTA, and 25 mM, 10-phenantroline) to block renin, ACE, and angiotensinases. The samples were frozen at −70°C immediately after collection. Some ocular fluid was frozen separately, without inhibitor solution, to study the metabolism of ^{125}I-ANG I and to measure renin substrate and albumin.

In most patients, a peripheral venous blood sample was drawn simultaneously with the collection of ocular fluid. Blood for angiotensin measurements (10 ml) was collected in prechilled tubes containing 0.5 ml of the above inhibitor solution and centrifuged at 3000*g* for 10 minutes at 4°C. Plasma was stored at −70°C and assayed within 2 weeks. Blood (10 ml) for measurements of renin substrate and albumin and for studies of the proteolytic breakdown of ^{125}I-ANG I was collected in tubes containing 0.1 volume of 0.13 M trisodium citrate. The blood was immediately centrifuged at 3000*g* for 10 minutes at room temperature, and plasma was stored at −70°C.

Because of the relatively low angiotensin levels and the small size of most ocular fluid samples (less than 0.5 ml), angiotensin measurements in ocular fluid were performed in pools (3 to 4 ml) consisting of 6 to 7 (subretinal and vitreous fluid) or 12 to 14 (aqueous fluid) samples. The recovery of angiotensins added to subretinal and vitreous fluid pools was 96% and 85%, respectively (n = 2 for each). Plasma levels of ANG I and II were measured in the individual samples. Renin substrate and albumin, both in plasma and ocular fluid, were also measured in the individual samples.

Breakdown of ^{125}I-ANG I in Human Ocular Fluid and Plasma. The metabolism of ^{125}I-ANG I was studied in pools (2 ml) of either subretinal fluid, vitreous fluid, or plasma obtained from subjects without diabetes. After the ocular fluid and plasma pools had been brought to a temperature of 37°C in a waterbath, the experiment was started by adding (at t = 0) 50,000 cpm of ^{125}I-ANG I (in 50 μl of Tris HCl buffer, pH = 7.4). Experiments were carried out in the presence or absence of the ACE inhibitor captopril (final concentration in the sample, 0.4 mM). Aliquots of 400 μl were taken at various times (at 0, 1, 2, and 5 minutes for plasma, at 0, 5, 10, and 20 minutes for subretinal fluid and at 0, 30, and 60 minutes for vitreous fluid) and immediately mixed with 50 μl inhibitor solution. The samples were kept on ice, and SepPak extraction was performed within 1 hour. The SepPak extracts were applied to the HPLC column, and ^{125}I-ANG I and ^{125}I-ANG II were measured by gamma counting as described before. Results are expressed as a percentage of total cpm in the SepPak extractions.

Data Analysis

Plasma proteins enter the vitreous mainly by diffusion.[24,25] One of the reasons the concentrations of these proteins are low in vitreous fluid is that they have to cross a relatively impermeable barrier. Breakdown of this blood-retinal barrier (BRB) leads to increased diffusion of plasma proteins into the eye. The rate of diffusion of a given protein is related to its molecular size and plasma concentration. Indeed, the concentrations of various plasma proteins relative to the concentration of albumin are similar in plasma and vitreous fluid,[7,24,25] provided they have approximately the same molecular weight. Thus, one would expect a relatively high intraocular albumin concentration (due to partial breakdown of the BRB) to be accompanied by a

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proportionally high concentration of plasma proteins of comparable size (e.g., renin substrate), whereas proteins or peptides of smaller size (e.g., ANG I and ANG II) reach higher concentrations in the eye. Therefore, we chose to take the ocular fluid-to-plasma concentration ratio of albumin as an index of the integrity of the BRB, an abnormally high ratio being an indication of breakdown of this barrier. By multiplying this ratio with the level of a given protein in plasma, the level of this protein in ocular fluid can be estimated, assuming that, as mentioned above, this protein is transferred from the blood into the ocular fluid and vice versa by mechanisms that are qualitatively and quantitatively the same as those for albumin. For example, the calculation of the renin substrate concentration would be as follows:

$$[RS]_{oc} = [RS]_{pl}*[ALB]_{oc}/[ALB]_{pl}$$

in which RS denotes renin substrate, ALB denotes albumin, oc denotes ocular fluid, pl denotes plasma, and brackets denote the concentration. If our assumptions are correct, the calculated concentrations should be equal, or at least closely correlated, to the actually measured concentrations.

Angiotensin levels in ocular fluid and plasma are expressed as arithmetical mean and range. Because of nonlinear distribution, albumin and renin substrate in ocular fluid and plasma are expressed as geometric mean and range. Unpaired *t*-tests were performed to analyze differences between subjects with and without diabetes. Values were considered significant if $P < 0.05$.

RESULTS

ANG I and ANG II Levels in Porcine Eyes

The levels of ANG I and II in porcine plasma, vitreous and ocular tissues are given in Table 1. Figure 1 shows the results in a retinal pigment epithelium + choroid extract. Not only were ANG I and ANG II detected, but small amounts of ANG III were present as well (Fig. 1). Expressed per gram of wet weight, the ocular tissue levels of ANG I were lower than those in plasma. The ANG II levels in the neural retina and anterior uveal tract were similar to or slightly higher than those in plasma, whereas in the retinal pigment epithelium + choroid layer, ANG II was 3-fold higher than in plasma. The high tissue levels of ANG II were not the result of in vitro conversion of ANG I by ACE, nor was ANG I degraded by other enzymes during storage or

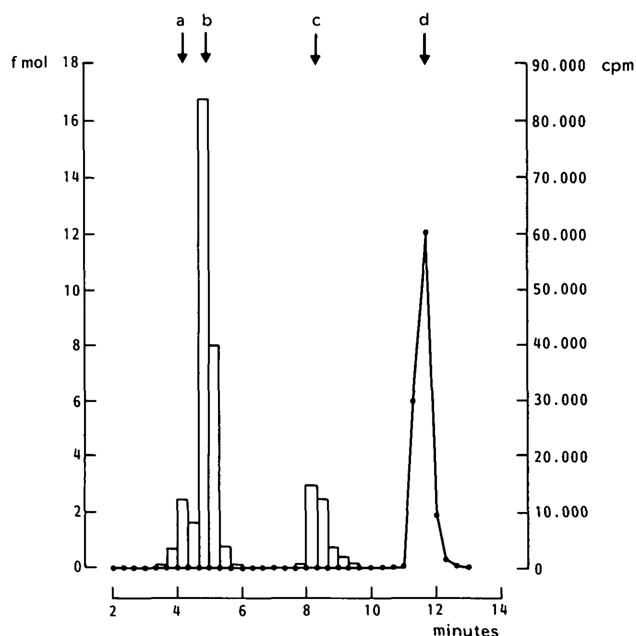

FIGURE 1. HPLC elution profile of endogenous angiotensins (bars) in a porcine retinal pigment epithelium + choroid tissue extract. ^{125}I-ANG I (closed circles) was added to measure breakdown during storage and purification (see Subjects and Methods for details). The retention times of standard angiotensins are given as a, ANG III; b, ANG II; c, ANG I; and d, ^{125}I-ANG I.

TABLE 1. Levels of Angiotensin I and II in Porcine Plasma and Vitreous and Ocular Tissues

	Angiotensin I (fmol/g)	*Angiotensin II (fmol/g)*	*Angiotensin II-to-I Ratio*
Plasma	9.7 (3.1–27.9)	7.5 (1.8–21.6)	0.8 (0.3–2.0)
Vitreous fluid	<1.1 (ND–3.1)	<0.9 (ND–1.3)	0.8 (0.1–4.0)
Neural retina	3.1	11.2	3.6
Retinal pigment epithelium + choroid	8.0	23.2	2.9
Anterior uveal tract	3.1	12.4	4.1

ND = not detectable.
Values are means and ranges of individual samples (plasma and vitreous fluid, n = 20) or means of 2 measurements in tissue pools (taken from 20 eyes each). Because the angiotensin I and II levels were below the detection limit in some vitreous fluid samples, the angiotensin II-to-I ratio in vitreous fluid was calculated using only those samples (n = 15) in which measurable amounts of angiotensin I and II were present.

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purification because, after the addition of ^{125}I-ANG I (either during storage or immediately before homogenization), only the intact radiolabeled peptide and not any of its metabolites was detected (Fig. 1). Previous studies, using RIA after extraction and HPLC separation, have documented that ^{125}I-labeled angiotensins show recoveries that are similar to the recoveries of the corresponding unlabeled peptides.[26,27]

In vitreous fluid, ANG I and ANG II levels were frequently close to or below the detection limit of the assay. Vitreous angiotensin levels were less than 10% of concomitant plasma angiotensin levels. The vitreous ANG II-to-I ratio, calculated in samples in which ANG I and II levels were above the detection limit of the assay, was similar to that in plasma, whereas the ANG II-to-I ratios in ocular tissues were clearly different (3- to 5-fold higher) from the plasma ANG II-to-I ratio.

^{125}I-ANG I Infusions in Rabbits

During the infusion, radioactivity in plasma increased with time, but the ^{125}I-ANG I levels reached a plateau within 15 minutes. The mean plateau concentration in the carotid artery (n = 3) was 550 (range, 165 to 1060) cpm ^{125}I-ANG I per milliliter of plasma. Expressed as a percentage of plasma radioactivity, the radioactivity in aqueous and vitreous fluid did not differ between samples from the first (t = 1 hour) and the second (t = 2 hours) eye. The radioactivity levels in aqueous fluid were 25.8% (range, 22.9% to 27.3%), and in vitreous fluid they were 2.4% (range, 1.6% to 3.5%) of those in concomitant plasma samples. HPLC separation of the radioactive components in ocular fluid revealed that virtually all radioactivity was present in the first few HPLC fractions. These fractions are known to contain small radiolabeled metabolites (predominantly ^{125}I-tyrosine).[19,28] Only very low concentrations of ^{125}I-ANG I were found in aqueous and vitreous fluid: 21 ± 24 cpm/ml and 6 ± 2 cpm/ml, respectively.

These findings demonstrate that during ^{125}I-ANG I infusion in normal healthy rabbits with presumably intact BRB, vitreous and aqueous fluid levels of ^{125}I-ANG I are reached that are approximately 1% and 4% of arterial plasma ^{125}I-ANG I levels.

ANG I, ANG II, Renin Substrate, and Albumin Levels in Human Ocular Fluid and Plasma

Plasma angiotensin levels were similar in subjects with and without diabetes (Table 2) and were not affected by either gender or the use of insulin (data not shown). In subjects without diabetes, the highest ocular angiotensin levels were found in subretinal fluid and the lowest (close to the detection limit of the assay) in aqueous fluid. ANG I and II levels in vitreous fluid of diabetic subjects were similar to those in vitreous of subjects without diabetes. Relative to ANG I, ANG II tended to be higher in vitreous from subjects with diabetes than in vitreous from subjects without diabetes, but the difference did not reach the level of statistical significance.

No differences in the albumin and renin substrate levels of vitreous fluid were found between subjects with and without diabetes (Table 3). The levels of albumin and renin substrate in plasma were also not different between the two groups (Table 3). Albumin and renin substrate concentrations in the ocular fluid compartments followed a similar pattern as the ANG I and II concentrations. The highest concentrations of albumin and renin substrate were found in subretinal fluid and the lowest in aqueous fluid. The vitreous fluid concentrations of albumin and renin substrate in subjects with diabetes using insulin were not significantly different from those in subjects with diabetes not using insulin (data not shown).

We also calculated the concentrations of renin substrate, ANG I, and ANG II in ocular fluids by using the formula presented in the Methods section and compared these calculated concentrations with the concentrations that were actually measured (Fig. 2). The calculation is based on the assumption that the

TABLE 2. Levels of Angiotensin I and II in Individual Plasma Samples and Ocular Fluid Pools from Diabetic and Nondiabetic Subjects

	n	*Angiotensin I (pM)*	*Angiotensin II (pM)*
Nondiabetic subjects			
Plasma	39	27.0 (1.9–86.6)	8.0 (0.7–20.7)
Subretinal fluid	3	24.5 (14.5–37.8)	5.4 (5.2–5.8)
Vitreous fluid	3	12.7 (7.0–17.2)	2.3 (1.3–3.0)
Aqueous fluid	2	2.0 (1.7–2.2)	0.5 (0.5–0.6)
Diabetic subjects			
Plasma	16	23.6 (3.9–94.7)	6.7 (0.9–24.9)
Vitreous fluid	4	8.7 (2.9–21.2)	4.4 (2.5–6.7)

Values are means and ranges. Ocular fluid pools were prepared by combining approximately 6–7 (subretinal fluid, vitreous fluid) or 12–14 (aqueous fluid) individual samples.

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TABLE 3. Levels of Albumin and Renin Substrate in Plasma and Ocular Fluid of Diabetic and Nondiabetic Subjects

	n	*Albumin (g/L)*	*Renin Substrate (nM)*
Nondiabetic subjects			
Plasma	39	32.5 (24.1–40.9)	1038 (572–1667)
Subretinal fluid	21	3.05 (0.35–29.6)	99 (21–963)
Vitreous fluid	18	1.25 (0.12–20.3)	42 (5–643)
Aqueous fluid	26	0.24 (0.06–0.45)	6 (2–15)
Diabetic subjects			
Plasma	16	31.1 (22.9–38.4)	937 (557–1280)
Vitreous fluid	24	1.87 (0.84–14.6)	58 (24–635)

Values are geometric means and ranges.

albumin ocular fluid-to-plasma concentration ratio is a measure of BRB integrity. It was found that the level of renin substrate in ocular fluid could be accurately predicted with this formula: (log[measured level] = 1.11 * log[calculated level] − 0.19; r = 1.00, $P < 0.05$). Not unexpectedly, in view of the much smaller size of ANG I and II, the ANG I and II ocular fluid-to-plasma concentration ratios were 10 times higher than the albumin ocular fluid-to-plasma concentration ratio (Fig. 2). The important point, however, is that, by taking the albumin ocular fluid-to-plasma concentration ratio as a basis for calculation, the calculated ANG I and II levels did correlate with the measured levels (log[measured level] = 0.99 * log[calculated level] + 0.94; r = 0.98, $P < 0.05$).

Breakdown of ^{125}I-ANG I in Human Ocular Fluid and Plasma

Incubation of ^{125}I-ANG I with pools of plasma, pools of subretinal fluid, and pools of vitreous fluid at 37°C resulted in proteolytic breakdown of the radiolabeled peptide (Fig. 3). The half-life of ^{125}I-ANG I in plasma, subretinal fluid, and vitreous fluid was 1.5, 14, and 134 minutes, respectively (mean of two experiments each). HPLC separation of the various metabolites showed formation of ^{125}I-ANG II (Fig. 4). In vitreous, only ^{125}I-ANG II and no other metabolites were found.

In the presence of the ACE inhibitor captopril, the breakdown of ^{125}I-ANG I was markedly reduced in

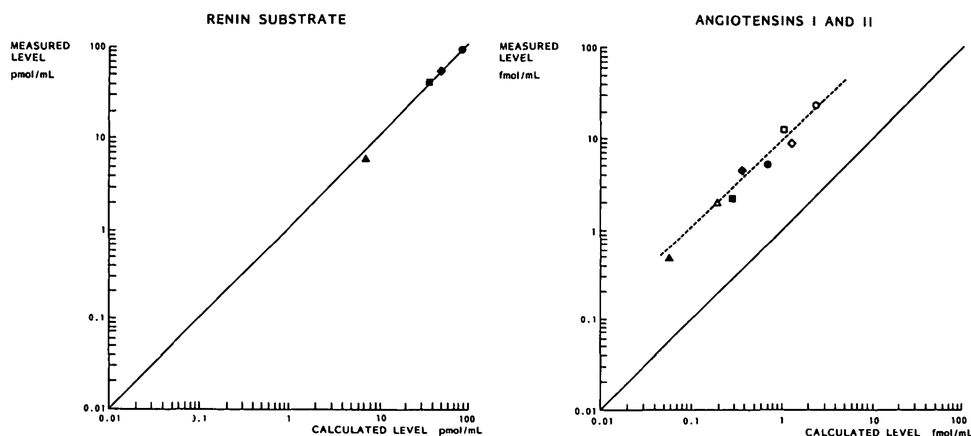

FIGURE 2. Measured versus calculated concentrations of renin substrate (left panel) and ANG I (open symbols, right panel) and ANG II (closed symbols, right panel) in subretinal fluid (circles), vitreous fluid (squares), and aqueous fluid (triangles) of subjects without diabetes and in vitreous fluid (diamonds) of subjects with diabetes. The line of identity is indicated. Calculated values were obtained as described under Data Analysis, taking the ocular fluid-to-plasma albumin concentration ratio as a measure of the integrity of the BRB. The measured levels of renin substrate were not different from the calculated levels. The measured levels of ANG I and ANG II were linearly correlated with the calculated levels (broken line; r = 0.98, $P < 0.05$) but were approximately 10 times higher. Data are compatible with the hypothesis that ANG I and ANG II and renin substrate in the ocular fluids are plasma-derived and diffuse through the BRB.

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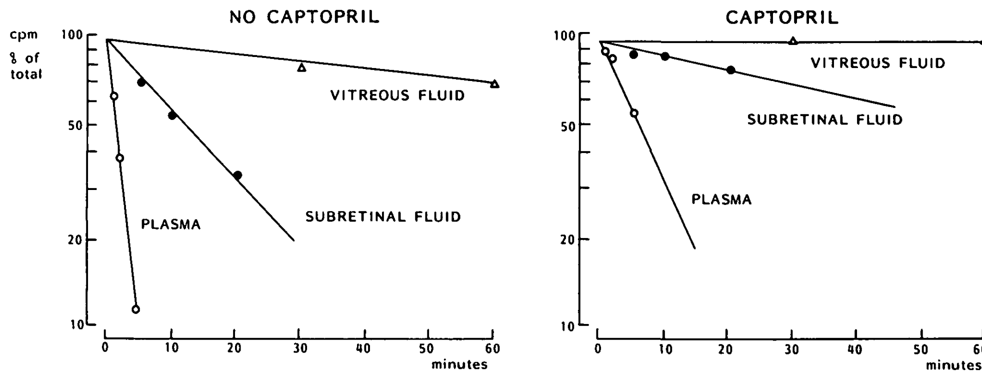

FIGURE 3. Breakdown of ^{125}I-ANG I in plasma, subretinal fluid, and vitreous fluid during incubation at 37°C in the presence (right panel) or absence (left panel) of the angiotensin converting enzyme inhibitor captopril. Data are means of two experiments.

subretinal fluid (half-life 87 minutes, n = 2) and plasma (half-life 5.4 minutes, n = 2), and it was completely inhibited in vitreous fluid (n = 2) (Fig. 3). With the addition of captopril, ^{125}I-ANG II was no longer present among the metabolites (Fig. 4), indicating that it was indeed ACE that was responsible for the formation of ^{125}I-ANG II in ocular fluid and in plasma. Based on the difference in half-life with and without captopril, it could be calculated that 70% to 80% of ANG I breakdown in plasma and subretinal fluid, and 100% of ANG I breakdown in vitreous fluid, was due to ANG I-II conversion by ACE.

DISCUSSION

The purpose of our study was to examine the presence of ANG I and ANG II in ocular tissues and fluids and to assess whether these angiotensins originate from local intraocular production or from diffusion from the blood compartment.

We found levels of ANG I and II in porcine ocular tissues (neural retina, anterior uveal tract, and retinal pigment epithelium + choroid) that were too high to be explained by contamination with plasma. Expressed per gram of wet weight, ocular ANG II levels were similar to or higher than plasma ANG II levels, whereas ocular ANG I levels were 30% to 80% of plasma ANG I levels. Previous studies in bovine eyes[8] have shown that the blood plasma content of the retina, anterior uveal tract, and retinal pigment epithelium + choroid are 1%, 5%, and 20% of total tissue weight, respectively. One may assume that the plasma content of porcine ocular tissues is similar. Thus, the angiotensin levels in the ocular tissues are 5- to 100-fold higher than can be explained by contamination of these tissues with blood. Moreover, the angiotensin levels in these tissues are unrelated to their expected plasma content. The present data are in agreement with measurements of prorenin, renin, and ACE in ocular tissues reported by us and others,[8,9,29] and with the fact that the renin-, renin substrate-, and ACE-genes are expressed in these tissues.[11] Local synthesis of angiotensins is also supported by the fact that the differences in angiotensin concentrations between the various ocular tissue layers resemble similar differences in both renin[8] and renin mRNA concentrations[11] between these tissues. The highest concentrations of renin, renin mRNA, ANG I, and ANG II were

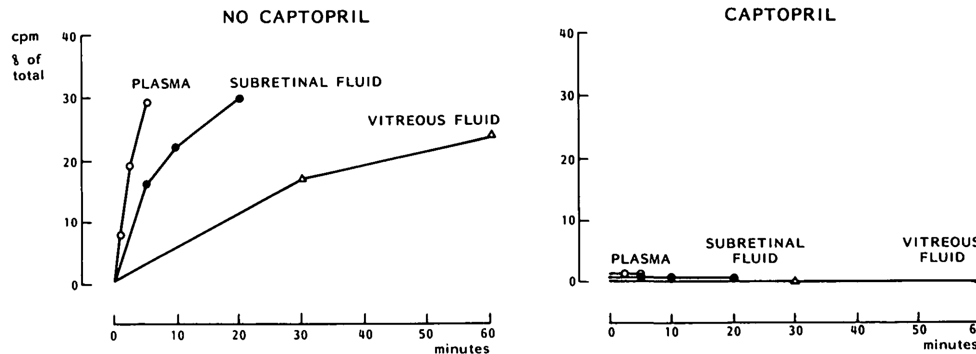

FIGURE 4. Conversion of ^{125}I-ANG I to ^{125}I-ANG II in plasma, subretinal fluid, and vitreous fluid during incubation at 37°C in the presence (right panel) or absence (left panel) of the angiotensin converting enzyme inhibitor captopril. Data are means of two experiments.

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found in the retinal pigment epithelium + choroid layer. The ANG II-to-I concentration ratio in porcine ocular tissues was 3- to 5-fold higher than the ANG II-to-I ratio in plasma. This has also been reported for other tissues.[30,31]

The levels of ANG I and II in porcine ocular fluid were low to undetectable, which contrasts with the relatively high levels in the surrounding ocular tissues. The absence of any significant overflow from the ocular tissues into vitreous fluid suggests a high degree of compartmentalization of the ocular renin-angiotensin system. In addition, the low angiotensin levels in vitreous fluid compared to plasma in these healthy, normal eyes are evidence for a highly efficient BRB for these peptides.

The BRB for angiotensin was more specifically studied by infusion experiments in rabbits. During a 2-hour systemic intravenous infusion of ^{125}I-ANG I, the concentration of ^{125}I-ANG I that was reached in vitreous fluid was about 1% of the concomitant level in arterial plasma. The level in aqueous fluid was in a similarly low range (4% of the arterial plasma level). We assumed that during the 2-hour infusion, an equilibrium between ^{125}I-ANG I in plasma and ocular fluid had been reached, given the fact that fluorescein, which has a molecular weight 3- to 4-fold lower than the angiotensins, reaches the vitreous within minutes of intravenous administration.[32-34] In normal subjects, ocular fluorescein levels are usually below 1% of concomitant plasma levels. Others have shown that neither ANG I nor ANG II is able to pass the blood-brain barrier.[35,36] This barrier is comparable to the BRB.[37] Microperoxidase, which has a molecular weight in the same range as ANG I and II, does not pass the BRB in normal rats.[38] Our data were obtained in rabbits, and, although species differences may exist, it appears that little, if any, plasma ANG I and II can cross the BRB in normal eyes. Partial breakdown of this barrier, however, may facilitate diffusion of angiotensins from the blood into the eye. Leakage of fluorescein into the eye is known to be increased in diabetic rats compared with normal rats,[39] and a direct correlation has been described between fluorescein leakage and breakdown of the BRB.[40]

In light of these animal data, it was not unexpected that ANG I and II could be readily measured in ocular fluid samples obtained from diseased eyes of patients with and without diabetes undergoing eye surgery. We measured the highest angiotensin levels in subretinal fluid and the lowest in aqueous fluid. Renin substrate and albumin could also detected in these ocular fluids, and their concentrations were also in the order: subretinal fluid > vitreous fluid > aqueous fluid. The ANG I, ANG II, renin substrate, and albumin concentrations in ocular fluid were not different between subjects with and without diabetes.

The concentration of plasma proteins in the vitreous fluid of normal, healthy human eyes has been reported to be only 1% to 2% of concomitant plasma levels.[24,25] We found higher levels in vitreous fluid for albumin and renin substrate (5% of plasma levels) in subjects with and without diabetes, presumably due to partial breakdown of the BRB. By taking the ocular fluid-to-plasma concentration ratio of albumin as an index of BRB integrity, and by multiplying this value with the plasma level of renin substrate, an accurate prediction of the renin substrate concentration was obtained (Fig. 2), confirming earlier data.[7] This suggests that renin substrate in ocular fluid is largely plasma-derived. Albumin and renin substrate have similar molecular weights (69 K and 65 K, respectively) and may therefore be expected to diffuse through the BRB to a similar degree.[7]

The levels of ANG I (molecular weight, 1.3 K) and ANG II (molecular weight, 1.0 K) in the ocular fluid samples were approximately 10 times higher than calculated on the basis of the albumin content of these samples (Fig. 2). This may be due to the smaller size of angiotensins, causing them to diffuse more easily through the BRB. In favor of leakage from plasma is our finding that, in human ocular fluid, the levels of ANG I and II correlated with the level of serum albumin (Fig. 2). Our explanation that the much smaller size of angiotensins can account for their relatively high levels in ocular fluid as compared with albumin is supported by an earlier observation that the levels of IgG (which has a higher molecular weight than albumin) relative to albumin are lower in ocular fluid than in plasma.[7]

Leakage of angiotensins from the surrounding tissues in these diseased eyes might also have contributed to the relatively high angiotensin levels in human ocular fluid. However, this seems unlikely in view of the fact that the ANG II-to-I ratio in ocular fluid was similar to that in plasma, whereas the ANG II-to-I ratio in tissues was higher than in plasma. If leakage from tissue sites had been important, one would expect the ANG II-to-I ratio in ocular fluid to be similar to that in the surrounding tissue, the more so because the rate of angiotensin breakdown in ocular fluid was low.

Alternatively, synthesis of angiotensins within ocular fluid might have contributed to its angiotensin content. This issue was addressed by measuring the proteolytic breakdown of ^{125}I-ANG I in human vitreous and subretinal fluid. ANG II appeared to be the most important breakdown product of ANG I. Its formation could be completely blocked by captopril, indicating the presence of ACE in ocular fluid. By combining these data on conversion and degradation of angiotensins with previously reported data on renin and renin substrate levels in human ocular fluids,[7] we could calculate that the measured levels of ANG I and II were several times higher than could be accounted for by in situ production in the ocular fluids.

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In summary, it appears that ANG I and II are produced locally in ocular tissues and that little of these locally produced angiotensins leaks into the ocular fluids. Only when the BRB is disrupted can angiotensins reach the vitreous fluid compartment in concentrations high enough to be detected by our assays. These data are in agreement with earlier findings that ocular tissues contain both renin and its inactive precursor prorenin,[8] whereas ocular fluids contain prorenin and little or no renin.[7,8] Cultured bovine theca cells[41] and rat adrenal glomerulosa cells[42] have also been reported to release only prorenin and no renin into the medium, whereas the cells contain both prorenin and renin.

The function of an ocular renin-angiotensin system is not yet clear. ANG II may regulate intraocular vascular tone and aqueous fluid hemodynamics.[16,17] In addition, ANG II has trophic and mitogenic actions on vascular smooth muscle and other cells.[43,44] We found prorenin to be increased in vitreous fluid from patients with proliferative diabetic retinopathy,[7] suggesting that an activated intraocular renin-angiotensin system may be involved in the development of this type of retinopathy.

Key Words

angiotensin I, angiotensin II, renin, diabetes, ocular renin-angiotensin system

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