Regulation of the renin expression in the retinal pigment epithelium by systemic stimuli

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Submitted 7 October 2009; accepted in final form 27 May 2010

THE RETINA EXPRESSES A LOCAL renin-angiotensin system (RAS). The expression of angiotensinogen, renin, angiotensin-converting enzyme inhibitor enalapril in mice increased the renin expression in both the kidney and the retina. Systemic infusion of ANG II led to a decrease in the renin expression in the kidney and in the retina and RPE. The ANG II-dependent down-regulation of renin expression in the RPE was prevented by systemic application of the AT1 receptor blocker losartan. However, water deprivation lead to an increase of the renin expression in the kidney but unexpectedly to a decrease of the renin expression in the retina. In sections of the mouse retina, the ANG II receptor AT1 was found in the RPE and localized at the blood side of the epithelium. Short-time cultured RPE cells showed increases in intracellular free Ca2+ in response to stimulation by ANG II that were sensitive to losartan. In summary, we conclude that the renin expression in cells of the blood/retina barrier is influenced by the systemic RAS. ANG II circulating in the plasma is likely a mediator of this influence.

MATERIALS AND METHODS

RT-PCR. To show the expression of angiotensin receptors and renin isoforms in the freshly isolated retina, RPE, and kidney, total mRNA was isolated from these tissues using the using the NucleoSpin RNAII kit (Macherey-Nagel, Düren, Germany), while reverse transcription was carried out using the RevertAid M-MuLV reverse transcriptase (Fermentas, St.Leon-Rot, Germany). Gene-specific PCR was performed using the primers listed in Table 1.

Modulation of renal renin expression in mice. Animals were maintained and bred in accordance with the Institutional Animal Care and Use Committee at University of Regensburg and the Association for Research in Vision and Ophthalmology statement for the use of animals in vision research. All animal experiments were formally approved by the German authorities. Adult FVN/B mice were obtained from own breeding. One group (n = 12) received the ACE inhibitor enalapril (10 mg kg−1·day−1) in drinking water for 6 days, while the other group (n = 12) received no treatment. For water deprivation, mice were kept under water deprivation for 24 h. To increase plasma ANG II, AT2 was infused by subcutaneous implanted minipumps for 3 days as previously published (30). At the end of the
experiment animals were killed by cervical dislocation, and retina, RPE, and kidneys were snap-frozen at −80°C until total RNA was isolated. To analyze a possible role of ANG II in mediating the effects of ANG II infusion, mice received losartan before and during ANG II infusion using the following protocol: BL6 mice received losartan (10 mg·kg⁻¹·day⁻¹) in drinking water for 6 days. On the third day, mice received ANG II infusion as described above while still receiving losartan. Control mice also received losartan alone and were injected with PBS.

**Real-time quantitative RT-PCR to measure the rate of renin expression.** Tissue samples of the kidney, the retina, and the RPE from treated and untreated mice were collected and subjected to homogenization and mRNA isolation. mRNA isolation and cDNA generation were performed using the same method as described for RT-PCR. For quantification, the SYBR-green based 2^ΔΔCt method was performed using the same method as described for RT-PCR. Total RNA from treated and untreated mice were collected and subjected to homogenization. Oligonucleotide primer pairs spanning an exon/exon boundary used in the study are listed in Table 1. Normalization of gene expression was achieved according to method described by Vandesompele et al. (34). β-Actin was used as a housekeeping gene for the calculation normalization factor in each tissue. To verify specificity of PCR amplification, dissociation curves were generated by gradually increasing the temperature from 60 to 94°C with ramping steps with 1°C per 10 s.

**Immunohistochemistry.** Mouse eyes were fixed with 4% (wt/vol) paraformaldehyde for 1 h at room temperature. After three washing steps with 1× PBS, eyes were cryopreserved using 30% (wt/vol) sucrose solution in PBS. Eyes were frozen in Tissue Tec (Leica, Nussloch, Germany), and thin cryostat sections (10 μm) of the posterior pole were prepared and mounted onto polylysine-coated slides. Cryo-slices of the retina were used for staining with antibodies directed against ANG II receptors (ab47408; Abcam, Cambridge, UK), and renin (DavidsBio, Regensburg, Germany). Immunolabeling of mouse eyes and kidneys was performed as described previously (26). Porcine primary RPE cells were grown on a sterile glass coverslip. Approximately 2 wk after seeding, cells were washed with 1× PBS and fixed for 10 min at room temperature with 4% (wt/vol) paraformaldehyde. After three washing steps with 1× PBS, cells were permeabilized with blocking/permeabilization solution [10% (vol/vol) normal goat serum and 0.5% (vol/vol) Triton X-100 in 1× PBS] for 20 min. Cells were then labeled for 1 h with anti-bestrophin-1 mAb antibody (ab2182; Abcam, Cambridge, UK; diluted 1:1,000 in 2% normal goat serum and 0.1% Triton X-100 in 1× PBS). After three additional washing steps, cells were incubated for 1 h with secondary antibody conjugated with Alexa 546. (Invitrogen, Karlsruhe, Germany). Cells were mounted in confocal matrix (Micro Tech Lab, Graz, Austria) and examined on an Zeiss Axiosvert 135M microscope attached to a LSM 410 confocal laser scanning system (Carl Zeiss, Göttingen, Germany).

**Determination of retinal renin content.** Mice received the angiotensin-converting enzyme (ACE) inhibitor enalapril (10 mg·kg⁻¹·day⁻¹) in drinking water for 6 days. After anesthesia (75 mg/ml ketaset + 5 mg/ml xylazine), mice were perfused by PBS to remove blood from the eye (visual control in albino mice retina). Eyes were opened by circumferential incision along the ora serrata. Anterior parts of the eye were removed and the retina was isolated by a fine pair of forceps and quickly frozen. The tissue was homogenized and centrifuged. The renin content was measured by RIA using saturating concentrations of renin substrate (Byk & DiaSorin Diagnostics, Germany) as described previously (27, 35).

**Cell culture.** To gain insight into possible intracellular mechanisms of ANG II on RPE cells, primary cultures of porcine RPE cells were established. Porcine RPE cells maintain angiotensin receptor expression in culture and show a high degree in differentiation, as can be shown by uniform expression of the RPE-specific protein bestrophin-1. Porcine eyes were obtained from a local slaughter house and transported in ice-cold buffer. Under sterile conditions, the eyes were opened by a circumferential cut along the ora serrata. The anterior parts of the eye including the retina were removed. Sheets of RPE cells were transported in ice-cold buffer. Under sterile conditions, the eyes were opened by a circumferential cut along the ora serrata. The anterior parts of the eye including the retina were removed. Sheets of RPE cells were suspended into a single suspension and plated out onto glass coverslips. After 1–2 wk, the cells formed confluent monolayers of cobblestone-shaped epithelial cells. The purity and degree of differentiation were shown by uniform and homogenous expression of bestrophin-1 by means of immunohistochemistry.

**Measurements of intracellular free Ca²⁺.** To identify the functional presence of the angiotensin receptors in the RPE, measurements of intracellular free Ca²⁺ were conducted by Ca²⁺-imaging using the Ca²⁺-sensitive fluorescence dye fura-2. Confluent monolayers of cultured RPE cells incubated with the membrane permeable fura-2-AM (5 μM) for 45 min. Cells loaded with fura-2 were placed into a perfusion chamber on stage of an inverted fluorescence microscope equipped with Ca²⁺-imaging system. During the experiment, the cells were superfused by a ringer solution (in mM: 145 NaCl, 0.4 KH₂PO₄, 1.6 K₂HPO₄, 5 glucose, 1 MgCl₂, and 1.3 CaCl₂-glucuronate). Fluorescence of fura-2 was elicited by two excitation wavelengths of 340 nm and 380 nm, and emission was filtered with a 510-nm filter and detected by a cooled charged-coupled device camera (CoolSnap, Visirion Systems, Puchheim, Germany). Data were collected and
analyzed with MetaFlour software (Visitron Systems, Puchheim, Germany). After stabilization of the fluorescence signal, cells were stimulated by extracellular application of 10 nM ANG II. After washout of ANG II and recovery of the signal, cellular calibration was performed to calculate the concentration of intracellular free Ca²⁺ from the fluorescence ratio of fura-2 from the two excitation wavelengths according to Grinkievicz et al. (10). In detail, intracellular fura-2 was first depleted from Ca²⁺ changing the bath medium into a Ca²⁺-free medium containing the Ca²⁺ ionophore ionomycin (1 μM). After reaching the minimal fluorescence, the bath medium was switched back to Ca²⁺ containing Ringer solution with 1 μM ionomycin to saturate fura-2 with Ca²⁺ and to reach the maximal fluorescence ratio.

RESULTS

Expression of renin in RPE cells. Freshly isolated cells of the mouse RPE, retina, and kidney were analyzed by means of RT-PCR for renin expression. RPE and retina cells express renin 1A, which is the secreted splicing variant, and not renin 1B, which has protease activity but is not secreted (Fig. 1A). To get an insight to a possible systemic influence on the regulation of renin mRNA expression in the RPE, renin mRNA was quantified by means of real-time PCR in the mouse retina, RPE, and kidney. In this set of experiments, defined manipulations of the systemic RAS were examined (Fig. 1, B-D). Administration of the ACE inhibitor enalapril (10 mg·kg⁻¹·day⁻¹ for 6 days) was used to decrease the activity of systemic RAS (Fig. 1B). ACE inhibition stimulates the renin production in the kidney leading to up to fivefold increase in plasma renin (35). In this experiment, we observed an increase in the renin mRNA expression in the kidney by 334 ± 21% (P < 0.05 vs. control; n = 12) of the control value. The renin expression increased as well in the retina to 2,500 ± 34% (P < 0.05 vs. control; n = 12) of the control value. In this...
experiment, we also measured the renin mRNA expression separately in the neuronal retina and in the RPE. In the RPE alone, the systemic administration of ACE inhibitor enalapril increased the renin expression to 1,082 ± 13% (P < 0.05 vs. control; n = 12) of the control value. To test whether ANG II in the plasma itself has an effect on the renin mRNA expression in the retina, we directly infused ANG II by a microinfusion system into mice for 3 days (Fig. 1C). This maneuver results in a decrease in plasma renin concentration by 50–70% (17, 22, 29). In our experiments, under these conditions the renin expression decreased in the kidney to 28 ± 2.3% of the control values. In the RPE and retina, renin mRNA production was also strongly reduced. ANG II infusion reduced the renin expression in the RPE and retina to 18 ± 2 and 28 ± 2% of the control value, respectively. Mice infused with physiological saline solution (0.9% NaCl) for 3 days were used as a control.

To further support this hypothesis we used the AT₁ receptor blocker losartan. In mice that received losartan (10 mg·kg⁻¹·day⁻¹), renin mRNA expression in the kidney was upregulated (to 200 ± 14%). Renin mRNA expression in the RPE only tended to increase (to 125 ± 17%). When losartan was administered during ANG II infusion, the renin mRNA expression in the RPE was the same as under control conditions (to 95 ± 6%). In the kidney, the down-regulation of renin mRNA expression during ANG II infusion was also not influenced by AT₁R blockade, too (120 ± 7%).

As a systemic influence that can occur naturally, we kept mice under water deprivation for 24 h. This is known to increase the plasma renin concentration up to fourfold (13). Under these conditions, the renin mRNA expression was increased in the kidney to 190 ± 11% (n = 3; P < 0.05 vs. control) of the control value (Fig. 1D, left). In contrast to that the renin expression in the eye was reduced to 23 ± 4% (n = 3; P < 0.05 vs. control) of the control value.

Since the first data were all obtained by measurements of the renin mRNA, we also checked whether the RPE expresses renin protein (Fig. 2). For this purpose, cryosections of the mouse retina were stained with an antibody against renin. In the kidney, the antibody clearly stains the juxtaglomerular cells in the afferent arteriole (Fig. 2, A and B). This staining was intensified in animals that received enalapril (Fig. 2B). In the sections of the mouse retina, renin protein was barely detectable under control conditions (Fig. 2C). In the retina of mice treated with enalapril, now a clear signal in the RPE layer was detectable (Fig. 2D). The detection of renin above the nuclei in the RPE indicates apical localization. In the inner parts of the retina, we did not detect renin protein. To verify that renin is secreted to the retina, the protease activity of renin was measured by RIA. Mice received enalapril for 6 days before the measurement of renin activity. Under these conditions renin activity was measured in the retina as ANG I production.

Fig. 2. Immunohistochemistry of cryosections of the kidney and the retina. A: expression of renin (green) and smooth muscle actin (SMA; red) in the kidney of control mice. G, glomerulus. Arrow shows renin labeling in close proximity to the afferent arterioles. B: expression of renin (green) and SMA (red) in the kidney of mice treated with enalapril. Arrow shows renin labeling in the afferent arterioles. C: expression of renin (green) in retinae of control mice. Differential interference contrast (DIC) image on the right depicts the retinal layers as outer plexiform layer (OPL), outer nuclear layer (ONL), and retinal pigment epithelium (RPE). Nuclei were counterstained with DAPI. Under control conditions, there was no renin protein detectable in the RPE by immunohistochemistry. D: expression of renin (green) in retinae of mice treated with enalapril. Under these conditions, renin was detectable in the RPE. Renin is localized to the apical side of the RPE cells. DIC image on the right depicts the retinal layers as OPL, ONL, and RPE. Nuclei were counterstained with DAPI. E and F: renin activity in the retina: Renin was measured in the retina of mice that were treated with enalapril to stimulate renin production in the RPE. Renin activity in the retina was measured by RIA and is given as ng ANG 1·h⁻¹·retina⁻¹. E: time dependence of renin activity. ANG I production increased with time of incubation. F: renin content of the retina as renin activity after 6-h incubation.
ANG I production increased with time of incubation. The activity was $24.4 \pm 9.4$ ng ANG I·h$^{-1}$·retina$^{-1}$ ($n = 4$).

Angiotensin receptors in the blood/retina barrier. The first set of experiments showed renin is expressed in the RPE and that this expression appeared to be modulated by plasma ANG II. Therefore, the functional expression of angiotensin receptors in the retina was investigated (Fig. 3). By means of RT-PCR, we detected the expression in the mRNA for AT$_{1A}$ and AT$_{1B}$ in the mouse retina as well as in the RPE (Fig. 3A). Furthermore, cryosections of the mouse retina were stained with antibodies against AT$_1$. To test the antibody, cryosections of the kidney of wild-type mice and of AT$_{1A}$/AT$_{1B}$ receptor double knockout mice were stained with antibodies against the AT$_1$ receptor (Fig. 3B). Since the antibody used here showed no staining in the knockout mouse, it is specific against AT$_1$ receptors.

In the wild-type mice, the antibody used here stained strongly the cells of the glomerulus, whereas in the knockout mice no staining was detected. Using this antibody, we stained the mouse retina (Fig. 3C). AT$_1$ receptor protein was found in the photoreceptor layer, in the layer of bipolar cell synapses, in the synaptic layers of the inner plexiform layer, and in the cell bodies of ganglion cells. Strong AT$_1$ receptor staining was also found in the RPE layer at the basolateral side of the epithelium, which faces the blood vessels of the choroid (Fig. 3D).

To demonstrate that RPE cells can react to ANG II, RPE cells of the porcine retina were kept in short-time culture (Fig. 4A; purity was confirmed by antibody staining against bestrophin-1, a RPE-specific gene), and cytosolic free Ca$^{2+}$ was measured by Ca$^{2+}$ imaging method (Fig. 4, B and D). Cultured RPE cells expressed renin and AT$_1$ receptor. Application of ANG II (10 nM) led in RPE cells to a biphasic rise in intracellular free Ca$^{2+}$: from a resting Ca$^{2+}$ level of 80 $\pm$ 4 nM, ANG II led to a Ca$^{2+}$ of 700 $\pm$ 45 nM, which was followed by a sustained Ca$^{2+}$ elevation at 160 $\pm$ 12 nM. These effects could be blocked by the presence of the AT$_1$ receptor.
blocker losartan. Losartan (10 μM) was applied for 2 min before additional application of ANG II (10 nM). Under these conditions, ANG II failed to induce increases in intracellular free Ca\(^{2+}\) (Fig. 4, C and D). In the presence of losartan, the application of ANG II led to a mean level of intracellular free Ca\(^{2+}\) of 111.8 ± 14.4 nM (\(P < 0.001\), against ANG II peak alone; no significance to base level; \(n = 14\)).

**DISCUSSION**

In this study, we provide for the first time evidence that the RPE expresses renin and that renin expression in the RPE can be modulated by changes in the systemic RAS. Thus the retinal RAS could be influenced by changes in the systemic RAS.

We found in freshly isolated RPE cells the expression of renin mRNA by RT-PCR and could demonstrate the presence of the renin protein in these cells in sections of the retina by means of immunohistochemistry and demonstration of renin protease activity by means of RIA. So far, the expression of renin was only showed in RPE/choroid preparations in which one cannot differentiate between the cell types that do express renin (36). With our work, we can confirm the expression in the RPE. Furthermore, we found that the RPE expresses the mRNA for the isoform renin 1A, which is the secreted form (25). The isoform renin 1B, which stays in the cytosol, is not expressed. The secretion of renin can occur to the retinal side of the epithelium or to the choroidal side of the epithelium. The RPE secretes renin to the retinal side. Renin protein was detected at the apical side of the RPE in cryosections of the retina. Furthermore, in enalapril-treated mice we could measure renin protease activity in the retina. Thus renin is not only expressed by the RPE it is also secreted by the RPE.

To study modulators of the renin expression in the RPE, we tried to identify possible systemic influences. An influence that can naturally occur is water deprivation. In mice kept under these conditions, the renin mRNA expression in the kidney was increased, whereas in the retina it was decreased. As a further systemic intervention, we used the systemic application of ACE inhibitor enalapril. In this case, the renin mRNA expression and protein were increased in kidney as well as in the retina. In this experiment, we could also demonstrate the
increase in the mRNA renin expression in the separated RPE and the retina. The effect in the retina was somewhat stronger and demonstrates that there are possibly more sources of renin in the whole retina. The changes in the renin expression in the retina possibly occur in the Müller cells, which were also identified as renin-producing cells (1). Our data could not confirm Müller cells as renin-producing cells. In sections of the mouse retina, we found no renin protein in the inner retina which could indicate renin production by Müller cells. The effect of enalapril in the RPE could also be demonstrated on the protein level. In retinal sections of the mouse stained against renin, renin was barely detectable under control conditions but showed a strong signal in enalapril treated mice. Furthermore, we could show the renin protease activity by means of RIA. Regarding the renin protein content, the renin production in RPE cells seems to be under control conditions virtually absent. However, renin mRNA production under control conditions could be further decreased by ANG II infusion. Thus there is under control conditions a physiological renin mRNA production that leads to no renin protein production or to a renin protein production that was too small to be detected by immunohistochemistry. This very low renin production under control conditions could indicate that activation of the intraocular RAS occurs only under strong modulations of the systemic RAS.

Systemic application of an ACE inhibitor is known to a decrease the ANG II concentration in the plasma (35), and this likely leads to an increase in the renin expression in the retina. The inverse relationship between the ANG II concentration in the plasma and the regulation of renin mRNA expression in the RPE suggests that ANG II could be the mediator of these regulatory effects on renin expression. Thus, in the next set of experiments, the effects of ANG II were studied directly. Infusion of ANG II in mice nearly resulted in a shut off of the renin mRNA expression in the RPE, resulting in an expression level lower compared with that under control conditions. Thus changes of the systemic RAS lead to changes in the renin expression in the RPE that forms a part of the blood/retina barrier. It is possible that ANG II is the mediator of the systematically controlled differential expression of renin in the RPE. Therefore, the role of ANG II receptors in the systemic modulation of intraocular RAS was investigated by systemic application of the AT1 receptor blocker losartan during ANG II infusion. It is known that application of losartan alone leads to a decrease in blood pressure and an increase in plasma ANG II concentration (20). Losartan did not change, while enalapril inhibited, the renin expression in the RPE. Both substances reduce the arterial blood pressure, but plasma ANG II is increased by losartan and decreased by enalapril (4, 19). Therefore, a decrease in blood pressure is unlikely to be a systemic mediator of renin expression in the RPE. In addition, it is known that the blood circulation in the eye has large autoregulative capacity. In experiments studying the effects of ACE inhibitor on the electroretinogram of healthy subjects, it was shown that the effects were independent from changes in blood pressure (16). Altogether, these earlier findings and our present data strongly suggest that circulating ANG II acting on AT1 receptors in the RPE is responsible for the shut off of renin expression in the retina in vivo.

To collect further evidence for a possible role of ANG II as a mediator of systemic influences on renin expression ANG II-dependent signaling in the RPE was studied. RPE cells in short-time culture showed increases in intracellular free Ca$^{2+}$ in response to application of ANG II, which were sensitive to the ANG II receptor blocker losartan. Thus RPE cells functionally express ANG II receptors. The ANG II-stimulated signal consists of an initial peak followed by a sustained elevation. Thus, as long as ANG II is present, it maintains a stimulation of RPE cells. With the use of stainings of retina sections of the mouse with antibodies against AT1, the ANG II receptor could be demonstrated in situ and could be localized in the basolateral membrane of the RPE. The antibody used here was tested in the kidney of wild-type mice and AT1 receptor double knockout mice. The antibody failed to stain glomerular cells in the knockout mouse, indicating the antibody used here is highly specific for the AT1 receptor. Thus the RPE is in vivo stimulated by ANG II rather from the blood side and not from the retinal side. Also, the effects of infusion ANG II could be explained if ANG II would reach the RPE by the blood stream and decrease the renin expression in the RPE. Since the ACE inhibitor likely decreases ANG II in the plasma and water deprivation likely increases ANG II in the plasma, the effects on renin expression in the mouse retina were also possibly mediated by changes in the ANG II concentrations in the plasma.

Among other effects (21), water deprivation is known to increase plasma renin concentration (13) and thus likely to an increase in the plasma concentration of ANG II leading to reduction of renin mRNA expression in the retina. However, in our study we cannot exclude vasopressin or sympathetic activation as further factors increasing the renin expression in the retina in response to water deprivation.

From these observations, we propose a hypothetical model. ANG II, which circulates in the blood stream, reaches the RPE through the blood vessels of the choroid. AT1 receptors in the RPE are located at the blood side and are stimulated by ANG II, which results in a decrease of renin expression.

With these observations, the RPE appears to be a part of the retinal RAS as well as of the systemic RAS. On one hand, it could possibly influence the RAS in the retina by renin secretion. On the other hand, it is able to react on changes in the systemic RAS. The systemic administration of ACE inhibitors to healthy subjects leads to the decrease of signals in the scotopic electroretinogram, indicating a decrease in the activity of rods and ON-bipolar cells as well as Müller cells and in the inner retina (14–16). Such a reaction could be mediated by AT1 receptors at the photoreceptors, synaptic layers of the retina and the ganglion cells that we detected by means of immunohistochemistry. In earlier studies (11, 12), ANG II appeared as a substance that decreased the activity of retinal neurons. Considering the observations in the present study, it is likely that the ACE inhibitor leads to higher renin expression in the RPE and subsequent increase in RAS activity in the retina leading to a decrease in retinal activity. Although changes of the intraocular RAS lead to modulation of neuronal activity in the retina (14, 16), the effects on visual function are so far not investigated by means of psycho-physical testing.

In summary, we showed that renin expression in RPE cells, which form a part of the blood/retina barrier, is regulated by changes in the systemic RAS. The regulation seems to be mediated by plasma ANG II though AT1 receptors in the RPE.
SYSTEnic RAS INFLUENCES REnIN EXPRESSIoN IN THE RPE

ACKNOWLEDGEMENTS

We thank Elfriede Eckert, Andrea Dannullis, Nestor Mas Gomez, Katharina Ehn, and Anna M’Bangui for expert technical assistance.

GRANTS

This work was supported by Deutsche Forschungsgemeinschaft DFG STR480/8–2 and SFB699.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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