Retinal Dysfunction in Diabetic Ren-2 Rats Is Ameliorated by Treatment with Valsartan but Not Atenolol

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PURPOSE. To determine whether diabetes leads to retinal neuronal dysfunction in hypertensive transgenic (mRen-2)27 rats (Ren-2), and whether the effect can be prevented by treatment of hypertension with either the angiotensin-1 receptor blocker (AT1-RB) valsartan or the β1-adrenergic receptor antagonist atenolol.

METHODS. Six-week-old Ren-2 rats were made diabetic (streptozotocin 55 mg/kg; n = 34) or remained nondiabetic (0.1 M citrate buffer; n = 43) and studied after 20 weeks. A subset of animals received valsartan (4 mg/kg per day) or atenolol (30 mg/kg per day) by gavage. Sprague-Dawley (SD) rats served as nonnormetensive controls for blood pressure (BP). We evaluated retinal function in all groups with a paired-flash electroretinogram over high light intensities (0.5–2.0 log cd·s·m⁻²), to isolate rod and cone responses.

RESULTS. A reduction in amplitude of all electoretinogram components (P111, P2, OPs, cone PII) was found in nondiabetic Ren-2 compared with nondiabetic SD rats. A further reduction was observed in diabetic Ren-2 rats. Treatment of both nondiabetic and diabetic Ren-2 rats with valsartan or atenolol reduced BP to within normal limits. This reduction produced some improvement in function in treated nondiabetic Ren-2 rats. However, in treated diabetic Ren-2 rats, retinal dysfunction was ameliorated by valsartan but not by atenolol, with a significant improvement (P < 0.05) observed in all components of the electroretinogram, with the exception of the OPs.

CONCLUSIONS. These findings suggest that hypertension induces retinal dysfunction that is exacerbated with diabetes and ameliorated by treatment with an AT1-RB, and not just by normalizing BP. These data provide further evidence for the importance of the renin-angiotensin system in development of diabetic complications. (Invest Ophthalmol Vis Sci. 2007;48: 927–934) DOI:10.1167/iovs.06-0892

Diabetic retinopathy is the leading cause of blindness in those of working age.1,2 It is largely considered a disease of the retinal vasculature whereby vessel integrity becomes increasingly compromised, as evidenced by breakdown of the blood–retinal barrier, neovascularization, and subsequent retinal contraction and detachment.3 In addition to the vascular lesions, there are functional and morphologic alterations in retinal neurons and glia during diabetes,4,5 which can occur before the onset of visible vascular disease.

It is well known that metabolic and hemodynamic factors are causative in the pathogenesis of diabetic retinopathy (reviewed in Wilkinson-Berka and Fletcher).6 Indeed, hypertension has been identified as a major independent risk factor for the development and progression of diabetic retinopathy in people with diabetes.7-9 Moreover, in diabetic patients without overt hypertension, retinopathy is associated with higher systolic blood pressure (SBP). Control of blood pressure reduces the progression of retinopathy by 35%.7 There are several possible explanations for the development or exacerbation of diabetic retinopathy in people with hypertension. Hypertension increases dilatation of retinal arteries by as much as 35%,9 and mechanical stretching can initiate intracellular signaling and alter secretion of numerous factors, including angiotensin II,10 endothelin-1, platelet-derived growth factor, and VEGF.11 Although there is compelling evidence that control of blood pressure reduces the progression of retinopathy, it is not clear whether a specific mechanism must be targeted to reduce the risk of progression.

The renin-angiotensin system (RAS) has been implicated in the progression of diabetic retinopathy,12 and angiotensin II is also a potent regulator of vessel patency and an important mediator of the development of hypertension. Within the retina, angiotensin II has been shown to induce changes in retinal blood flow by causing the contraction of retinal pericytes.13 Moreover, overactivity of the retinal RAS, which has been shown to be independent of the systemic RAS,14 promotes endothelial cell proliferation in ischemic retinopathies such as retinopathy of prematurity15 and diabetic retinopathy.16 Therefore, inhibitors of the RAS could play an important role in the treatment of those with diabetes, because of a reduction in blood pressure and also because of direct inhibition of the effects of angiotensin II within the retina.

Although the changes that occur in the retinal vasculature during diabetes have been well characterized, there is increasing evidence to suggest that retinal neurons are affected early in the disease. Apoptosis of retinal neurons,17,18 decreases in the number and length of photoreceptors,19 and functional deficits as early as 2 days after diabetes20 have all been demonstrated early in the experimental condition. In the present study, it is important to note that photoreceptor deficits in diabetic animals have been shown to be ameliorated by inhibiting the RAS with the ACE inhibitor perindopril.21 However, it is unknown whether this effect is due to the treatment of hypertension, or whether the improvement in function is due to a specific effect on the RAS.

In this study, we evaluate retinal function in transgenic Ren-2 rats using the electroretinogram (ERG). The ERG is used to evaluate neuronal and glial function in retinal diseases and has been used extensively in evaluating diabetic retinopathy changes.22-25 The transgenic Ren-2 rat exhibits fulminant hypertension due to the overexpression of renin and angiotensin II in extrarenal tissues26,27 and was developed by introducing...
the murine Ren-2 gene into the genome of the Sprague-Dawley (SD) rat.28 When made diabetic with streptozotocin (STZ), the transgenic Ren-2 rat develops severe microvascular disease in the kidney and eye.16,29 As such, the diabetic Ren-2 rat is a good model for the investigation of the interaction of hypertension and diabetes in the development of diabetic eye disease.

The aims of this study were twofold. First, we evaluated retinal function in the Ren-2 rat, to establish the effects of hypertension and diabetes on retinal neuronal function in this animal model. Second, we evaluated retinal function after two treatments of systolic hypertension: a specific angiotensin type 1 receptor blocker (AT1-RB), valsartan, to evaluate the effect of lowering blood pressure by specifically targeting the RAS system, and comparing these results to treatment with the β1-adrenergic receptor blocker, atenolol. In this way, it can be established whether antihypertensive therapy, per se, or specific inhibition of the RAS plays a role in the development of retinal changes in diabetes. Moreover, by using a component analysis of the ERG, we can identify the effects of hypertension, diabetes, and the hypertensive treatments on specific classes of retinal neurons. Evaluation of retinal function may provide a useful, clinically relevant tool with which to assess the efficacy of novel treatments.

**Materials and Methods**

**Animals**

A total of 77 animals were used in this study. Thirty-four, 7-week-old female hypertensive transgenic Ren-2 rats (homozygous for the Ren-2 gene), were rendered diabetic by a single injection of streptozotocin (STZ; 50 mg/kg; Sigma-Aldrich, St. Louis, MO) dissolved in 0.1 M citrate buffer (pH 4.5). Nondiabetic SD and nondiabetic Ren-2 rats received an injection of citrate buffer alone (n = 43). Diabetic animals received triweekly injections of insulin (4–6 units intraperitoneally; Ultratard, Novo Nordisk, Bagsværd, Denmark) to promote survival and weight gain and prevent ketoacidosis. Animals were randomized to receive water (n = 13 nondiabetic SD, 12 nondiabetic Ren-2, 13 diabetic Ren-2), valsartan (4 mg/kg per day) by gavage; n = 11 nondiabetic Ren-2, 11 diabetic Ren-2) or atenolol (30 mg/kg per day by gavage; n = 7 nondiabetic Ren 2, 10 diabetic Ren-2). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Metabolic indices measured on every animal included blood glucose, glycosylated hemoglobin, body weight, and SBP. Only diabetic animals with a blood glucose level ≥ 15 mmol/L were included in the study.

SBP was measured before the onset of diabetes (6 weeks of age; week 0) and 20 weeks after the onset of diabetes in conscious animals, via tail cuff plethysmography. Arterial pressure changes, detected by a pneumatic pulse transducer (PE-300; Nacro Biosystems Inc., Houston, TX), were recorded (Chart Program, ver. 3.5 on a MacLab/2E System; ADInstruments Pty. Ltd., Castle Hill, NSW, Australia). Measurements were taken at the same time of the day to minimize circadian influences; five consecutive measurements were necessary to reduce variability.

**Retinal Function: Paired-Flash Electroretinogram Recording**

Retinal function was assessed in all animals by measuring the flash electroretinogram (ERG) after 20 weeks of diabetes, as previously described.20 Briefly, after dark adaptation overnight, animals were anesthetized with a mixture of ketamine and xylazine (60:5 mg/kg), corneas were anesthetized with two drops of topical 0.5% proxymetacaine (Ophtalic; Allergan, Frenchs Forest, NSW, Australia), and pupils were dilated with 0.5% tropicamide (Mydriacyl; Allergan). Full-field flash ERGs were recorded with stainless-steel electrodes (active, cornea; inactive, mouth) referenced to a stainless-steel ground (26-gauge needle) inserted in the tail. Responses were amplified (gain × 5000; −13 dB at 1 Hz and 1 kHz; ADInstruments) and digitized at 10 kHz over a 200-ms epoch. A commercial photographic flash unit (Mecablitz 60CT4; Metz-Werke GmbH & Co. KG, Zirndorf, Germany) was delivered through a Ganzfeld sphere, and stimulus energy was attenuated by altering the flash aperture settings and implementing neutral density filters. Signals were collected over an ensemble of increasing light intensities (0.5–2.0 log cd·s·m−2). A paired-flash protocol was used to isolate cone and rod contributions of the ERG waveform as described in Phipps et al.20 In brief, two flashes were presented in succession with an interstimulus interval (ISI) of 0.8 seconds. The short ISI ensured that rod responses were not recovered once the second flash was presented, resulting in a cone-only response to the second flash. Rod contributions were isolated from digital subtraction of the cone response from the mixed response (signal from the first flash).

**ERG Component Analysis**

Photoreceptor function was assessed by modeling the leading edge of the a-wave (PII model). The PII model is based on the biochemical processes involved with phototransduction and in this study, a modified version of the Hood and Birch model31 was used:

\[
\text{PII}(i + t) = \left[1 - \exp(-i - S(t - t_d)^2)\right] \cdot R_{\text{max}}
\]

where PII gives the summed photocurrent as a function of luminous exposure \(i\) (cd·s·m−2) and time \(t\) (in seconds). \(R_{\text{max}}\) (in micromolts) is the saturated amplitude of the PII, \(S\) (sensitivity) represents the gain of the phototransduction process (m2·cd−1·s−1), and \(t_d\) (in seconds) is a brief delay that accounts for biochemical and recording latencies after stimulation. The PII model was fitted to an ensemble of a-waves (1.3–2.0 log cd·s·m−2) for each animal through the optimization of the \(R_{\text{max}}\) and sensitivity parameters. Optimization was accomplished through minimization of the sum-of-squares (SS) error term using the Solver Function of Excel (Microsoft, Redmond, WA).

Inner retinal function was examined by characterization of the PII. The PII component is represented by the b-wave after extraction of the PII from the raw rod waveform, and this was described by its maximum amplitude (in micromolts) and implicit time (in microseconds). Oscillatory potentials (OPs) appear on the rising slope of the PII and were isolated by removing elements that overlap with the dominant frequencies of OPs in the frequency spectrum (PII and PIII).32 In short, the PII and the rising slope of the b-wave were digitally subtracted from the raw waveform to yield oscillations that were filtered (55–280 Hz at −2.5 dB, 512-tap FIR filter, Blackman window). The resultant conditioned waveform was modeled by a Gabor (a Gaussian envelope multiplied by a sinewave carrier), in the time domain:

\[
\text{Gabor}(x) = a \cdot e^{\frac{-x^2}{2\sigma^2}} \cdot \sin2 \cdot bx \cdot \pi + \rho
\]

**Statistical Analysis**

Data were analyzed (SigmaStat for Windows, ver. 3.10; Systat Software Inc, Point Richmond, CA), and a one-way ANOVA with a Tukey post hoc comparison was applied, with P < 0.05 considered statistically significant for homogenous and normally distributed data. In cases of non-normal or nonhomogenous data, a Kruskal-Wallis test was applied with the Dunn post hoc comparison, and P < 0.05 was considered statistically significant.

**Results**

The results for glycated hemoglobin (HbA1c), SBP, and body weight are summarized in Table 1. Poorly controlled diabetes was confirmed in all diabetic animals by elevation in the level of HbA1c. In addition, untreated Ren-2 rats had elevated SBP, as previously described.29 Treatment with either valsartan or atenolol reduced SBP, so that there was no difference between
untreated nondiabetic SD rats and any of the treated Ren-2 rats. There was no statistically significant difference in SBP between the valsartan-treated animals and those treated with atenolol.

**Hypertension-Caused Dysfunction of Retinal Neurons in the Ren-2 Rat Exacerbated by Diabetes**

Figure 1A shows representative rod-derived waveforms of nondiabetic SD, nondiabetic Ren-2, and diabetic Ren-2 rats after 20 weeks of diabetes. A reduction in the waveform amplitudes of both the nondiabetic and diabetic Ren-2 animals is clearly apparent compared with the nondiabetic SD.

We quantified the change in amplitude of the different components of the ERG, as shown in Figure 1B. Two findings are apparent from the data. The first is that nondiabetic Ren-2 animals (unfilled bars) showed a significant reduction ($P < 0.05$) in all components of the ERG compared with nondiabetic SD rats (rod photoreceptor PIII, rod bipolar PII, OPs and cone PII), suggesting that hypertension causes retinal dysfunction in these animals. The second finding is that diabetic Ren-2 animals (gray bars) showed a further retinal dysfunction, with all components significantly worse than those in nondiabetic Ren-2 animals ($P < 0.05$), with the exception of the OP component ($P = 0.49$). The latency of the photoreceptorial and postreceptorial responses were also analyzed and found to be no different between any of the groups tested ($P > 0.05$, data not shown).

In summary, retinal function in both nondiabetic and diabetic Ren-2 rats was reduced after 20 weeks of diabetes compared with that in nondiabetic SD rats.

**Effect of Treatment of Hypertension on Retinal Function in Nondiabetic Ren-2 Rats**

Having established that retinal function is affected in the nondiabetic Ren-2 rat, we next examined whether treatments that reduce SBP could prevent neuronal dysfunction. Figure 2A shows representative waveforms of the a-wave of nondiabetic Ren-2 rats treated with valsartan or atenolol compared with an untreated Ren-2 nondiabetic animal, indicating an improvement in the photoreceptor function with both treatments in the nondiabetic Ren-2 animals.

The observation for single animals was confirmed by the group data shown in Figure 2B. In this figure, the 95% confidence intervals of the PIII amplitude in nondiabetic SD rats is shaded in light gray and the 95% confidence intervals for untreated nondiabetic Ren-2 rats is shaded in dark gray, to enable comparisons. A small but nonsignificant improvement compared with the nondiabetic Ren-2 animals (dark gray shading) was seen in the amplitude of the rod PIII in valsartan-treated animals. Nondiabetic Ren-2 rats treated with atenolol showed a larger improvement in PIII function ($P < 0.05$ compared with nondiabetic Ren-2 untreated animals), but still failed to reach the level of nondiabetic SD animals.

The effect of the treatment of hypertension on inner retinal neurons is shown in Figures 2C to 2H. Similar to the rod photoreceptor response, the rod bipolar cells (Figs. 2C, 2D) and the OP response (Figs. 2E, 2F) showed improvement with treatment with both valsartan and atenolol, but this improvement did not reach statistical significance. When a twin-flash paradigm was used to elicit the cone response, no change was observed in the function of the cone PII in either valsartan- or atenolol-treated animals (Figs. 2G, 2H).
In summary, treatment of hypertension improves the function of nondiabetic Ren-2 rats, with a significant improvement observed in the rod PIII response when treated with atenolol.

**Effect of Valsartan and Atenolol on Retinal Function in Diabetic Animals**

To investigate whether antihypertensive treatment, per se, or specific blockade of the RAS has an effect on the development of retinal dysfunction in diabetes, we evaluated the effect of valsartan and atenolol on diabetic Ren-2 animals (Fig. 3). In the figure, the 95% confidence limits of the diabetic Ren-2 animals is shown in dark gray, with the nondiabetic SD animals 95% confidence limits in light gray. A significant improvement (\( P < 0.05 \), Kruskal-Wallis test, Dunn post hoc) in the rod photoreceptor PIII amplitude was found in valsartan-treated diabetic animals compared with untreated diabetic Ren-2 animals (Figs. 3A, 3B). This significant improvement with valsartan was also found in the rod PII (\( P < 0.05 \); Figs. 3C, 3D) and the cone PII (\( P < 0.05 \); Figs. 3G, 3H). No significant improvement was seen in the diabetic Ren-2 OP component (Figs. 3E, 3F). No improvement in function was found in any component with the atenolol-treated diabetic Ren-2 animals compared with the untreated Ren-2 animals.
In summary, valsartan-treated diabetic Ren-2 animals showed significantly larger rod PIII, rod PII, and cone PII amplitudes than did untreated diabetic Ren-2 animals. The ERG amplitudes of atenolol-treated diabetic Ren-2 rats were unchanged compared with their untreated counterparts.

**DISCUSSION**

This study showed that the Ren-2 rat displays a neuronal dysfunction that is exacerbated with diabetes, developing a substantial loss of function in rod photoreceptors and neurons mediating the cone pathways. This loss in the diabetic Ren-2 rat can be ameliorated by treatment with valsartan but not atenolol. The results indicate that, whereas hypertension is a risk factor in the development of retinopathy changes, the treatment of hypertension alone may not be enough to prevent neuronal losses during diabetes. Rather, treatment with specific inhibitors of the RAS is necessary for preventing neuronal loss. This may have important implications for therapeutic prevention of diabetic retinopathy.
Neuronal Losses with Hypertension

We observed a reduction in all components of the ERG (PIII, OPs, and rod and cone PII) in the nondiabetic Ren-2 rat. The Ren-2 animal model is a transgenic rat into which the mouse Ren-2 gene has been introduced, and thus it displays fulminant hypertension33 and elevated renin in extrarenal tissues including the eye.33 In the retina, endothelial cell proliferation and VEGF expression is increased in the Ren-2 rat.16 These changes would likely lead to alterations in the neuronal function of these animals, but few studies have examined functional changes with hypertension. However, there is some evidence that the OPs of the ERG are reduced in hypertensive subjects without hypertensive retinopathy.34

Although we have found a decrease in function in the nondiabetic Ren-2 rat, it is unclear whether this effect was due to hypertension, per se, or to an elevated renin effect. Treatment of hypertension in the Ren-2 animals with both valsartan and atenolol produced improvements compared with the nondiabetic state. However, this effect was significant only for the PIII component of the atenolol-treated animals. This result is perplexing, in that valsartan and atenolol both reduced the SBP by similar amounts, and is difficult to interpret because of the confounding effects of angiotensin II and systolic hypertension on retinal function. Indeed, angiotensin II is a neuromodulator in the brain, and there is evidence that angiotensin II can modulate neural activity within the retina.35 Receptors for angiotensin II have been localized to amacrine cells, and both renin and angiotensin II are known to be localized to Müller cells.35,36 Although expression of AT1 and AT2 receptors on photoreceptors has not been described, angiotensin II is known to modulate calcium currents in ganglion cells.37,38 It is possible that atenolol acts on the retinal vasculature to improve PIII function by improving circulation. For example, β1-adrenergic receptor antagonists have antioxidant properties,39 an issue to be considered later. However, it is difficult to draw firm conclusions from our study about the independent effects of angiotensin II on SBP or retinal function without further experimentation.

Neuronal Changes with Hypertension and Diabetes

We observed a substantial reduction in the rod photoreceptor response in diabetic Ren-2 rats compared with nondiabetic Ren-2 and nondiabetic SD rats. In fact, all components, with the exception of the OPs, were significantly reduced in the diabetic Ren-2 animals compared with the nondiabetic Ren-2 animals. Many studies suggest that inner retinal function, especially the OP amplitude, is abnormal in diabetes.40,41 The nonsignificant finding for the OP component between the nondiabetic and diabetic Ren-2 states most likely reflects the substantial reduction in this component in the nondiabetic Ren-2 animals, which in turn reflects the vulnerability of the OPs to disease states.40 However, the large OP loss in both the nondiabetic and diabetic Ren-2 animals may also be the result of serial losses from the photoreceptor dysfunction evident in nondiabetic Ren-2 and diabetic Ren-2 rats. The ERG is a serial waveform, with losses in the a-wave translated into reductions in the b-wave and OPs. Our results show that the amplitude of the inner retinal responses, the rod PII and OPs, were reduced by the same degree as rod photoreceptor function (rod PII), suggesting that photoreceptors are the principal neuron affected by diabetes in the diabetic Ren-2 rat, with inner retinal losses occurring serially as a result of the photoreceptor reduction. These results confirm previous studies20–22 which demonstrated that rod photoreceptors are the primary retinal neuron affected by diabetes.

Effect of Valsartan and Atenolol on Diabetic Neuronal Losses

Our results indicate that treatment of diabetic animals with valsartan but not atenolol prevented neuronal losses in the diabetic Ren-2 rat, suggesting that it is through a specific effect on the RAS and not control of blood pressure alone that is necessary to prevent retinal dysfunction during diabetes. Moreover, our results provide further evidence of the importance of the RAS in the development of diabetic retinopathy changes. Angiotensin II is known to act on AT1 receptors expressed by pericytes and elicits contraction of these cells.15 Therefore, it is likely that valsartan treatment directly affects pericyte function. Moreover, antagonists of AT1 receptors and other blockers of the RAS are known to prevent neovascularization in oxygen-induced retinopathy.6,15 In the diabetic Ren-2 rat model, elevated VEGF expression and endothelial cell proliferation are prevented by treatments that inhibit the RAS.16 In addition, treatment with captopril but not atenolol prevents uptake of glucose into cultured retinal endothelial cells.45 With this in mind, it is possible that the improvement in neuronal function observed in this study is related to improvements in the retinal vasculature.

Apart from the specific effects of angiotensin receptor blocker on the retinal neurons, treatments of the RAS system have beneficial effects on the diabetic state in general. Treatment with AT1 receptor blockers decrease the risk for new onset diabetes in high-risk patients.44 Treatment with valsartan also has direct benefits over atenolol, with valsartan improving resistance in arteries in diabetic patients, while atenolol-treated patients had stiffer arteries.45 When considering the effects of the AT1 receptor blockade and treatment with a β1-adrenergic receptor antagonist, it is also useful to consider the anti-inflammatory effect that treatment with AT1 blockers has on the vasculature. There is some suggestion that by blocking the AT1 receptors, the AT2 receptors are relatively stimulated by circulating angiotensin II.46,47 The AT2 receptors have a number of functions, including the inhibition of inflammatory responses.48 Moreover, recent studies suggest that hyperglycemia-induced oxidative stress plays a role in the development of diabetic complications including diabetic retinopathy49,50 and that both angiotensin II and activated AT1 receptors produce intracellular oxidative stress (reviewed in Cierello51). Although valsartan treatment is likely to block this effect due to its action on AT1 receptors, there is also evidence that β-adrenergic antagonists, such as atenolol, have associated antioxidant properties and have some ability to scavenge oxygen and nitrogen species.52 As oxidative stress is implicated in the development of diabetic retinopathy, it is possible that both valsartan and atenolol act through this mechanism to improve retinal function. Indeed, the improvement seen in PIII function in atenolol-treated animals could be the result of atenolol’s antioxidant properties.

It is well established that blood pressure is an important risk factor in the development of diabetic retinopathy.53,54 Moreover, it has been demonstrated that the RAS has a role in the prevention of retinopathy in humans, with the ACE inhibitor lisinopril slowing the progression to proliferative retinopathy.55 The results of our study suggest that, although hypertension increases the effect of diabetes on retinal neuronal function, it is through its effects on the RAS, as normalization of blood pressure alone with the β-blocker atenolol has no effect on retinal dysfunction in diabetes. This finding is important in targeting investigations into the mechanisms of development of proliferative retinopathy.

In summary, our findings indicate that inhibiting the actions of angiotensin II with valsartan prevented the development of neuronal deficits in diabetes and that this effect was indepen-
dent of controlling hypertension. These results lend further weight to the HOPE55 and EUCLID56 studies, which suggest that blockage of the RAS is beneficial for preventing the development of complications in people with diabetes.

References


