Renoprotective and anti-hypertensive effects of combined valsartan and perindopril in progressive diabetic nephropathy in the transgenic (mRen-2)27 rat

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Abstract

Background. We have previously reported that severe glomerulosclerosis progressively develops in the streptozotocin (STZ) diabetic transgenic (mRen-2)27 rat. In this diabetic model, monotherapy with either angiotensin converting enzyme inhibition (ACEI) or angiotensin type 1 (AT₁) receptor blockade is largely renoprotective. The objective of the present study was to determine if a combination therapy at lower doses than monotherapy would confer greater renoprotection.

Methods. At 6 weeks of age, non-diabetic control and STZ diabetic female heterozygous Ren-2 rats were randomized to receive vehicle, the AT₁ receptor blocker valsartan (V, 20 mg/kg/day), the ACEI perindopril (P, 6 mg/kg/day), or a combination of low-dose V + P (V, 3 mg/kg/day plus P, 0.5 mg/kg/day) for 12 weeks.

Results. Systolic blood pressure was lowered with all treatments, but the greatest reductions were observed with V monotherapy and combination V + P therapy. All treatments reduced albuminuria, the decline in glomerular filtration rate, and cortical collagen staining, to the same extent. The glomerulosclerotic index was increased with diabetes and reduced with both V and P monotherapy. However, the low-dose combination therapy was more effective than single therapy and reduced severe glomerulosclerosis to levels observed in non-diabetic controls.

Conclusions. Monotherapy with either V or P reduced blood pressure and retarded the decline in renal function and glomerulosclerosis in the diabetic Ren-2 rat. Combination therapy has the additional benefit of requiring only low doses of AT₁ receptor blockade and ACEI to achieve superior renoprotective effects in this diabetic nephropathy model.

Keywords: angiotensin; diabetes; glomerulosclerosis; hypertension; (mRen-2)27 rat; renin

Introduction

The deterioration in renal structure and function associated with the progression of diabetes can be improved by blockade of the renin–angiotensin system (RAS), with either angiotensin type 1 (AT₁) receptor blockers or angiotensin converting enzyme inhibitors (ACEI) [1–4]. The renoprotection afforded by these agents has been attributed primarily not only to a reduction in systemic but also intraglomerular pressure [5]. Alternatively, RAS blockade may suppress a locally activated kidney RAS resulting in a decrease in proinflammatory and proscerotic cytokines which contribute to the progression of diabetic microvascular and tubulointerstitial disease [3,4].

Although the administration of ACEI results in a fall in plasma angiotensin II (Ang II) levels, the efficacy of ACEI is probably limited by their inability to completely block ACE activity and the generation of Ang II through other enzymatic pathways [6,7]. However, ACE inhibitors have other effects including interference with the breakdown of bradykinin [8]. Long-term ACEI use is associated with a return in circulating Ang II levels following a reactive rise in plasma active renin and angiotensin I due to the interruption of Ang II feedback on renin release [9]. On the other hand, angiotensin-receptor blockers (AT-RB) do not affect bradykinin production and should theoretically block the actions of Ang II chronically at the receptor level. Angiotensin receptors exist in two main forms, AT₁ and the angiotensin type 2 (AT₂) receptors. The
main target for AT-RB is the AT$_1$ receptor, which mediates Ang II-induced vasoconstriction and electrolyte homeostasis, whereas the AT$_2$ receptor is overexpressed in fetal tissues, where it may stimulate growth [10]. An unknown issue with AT$_1$ receptor blockade is whether chronic stimulation of AT$_2$ and any other Ang II receptors from a reactive rise in plasma renin, and possibly angiotensin I–7, has pathophysiological consequences [11]. This possibility in concert with the high level of Ang II generated following ACEI, justifies exploring the use of combination therapy for the treatment of chronic renal pathologies, since any AT-RB-induced rise in Ang II should be attenuated by parallel ACEI administration.

This concept was tested in the hypertensive transgenic (mRen-2)27 rat, which, when made diabetic with streptozotocin (STZ), displays severe glomerulosclerosis, tubulointerstitial disease, and a decline in renal function [3,4]. Using this model, we have previously shown that the ACEI perindopril and the AT$_1$ receptor blocker valsartan separately improve renal pathology [3,4]. The present study sought to determine if a low-dose combination of perindopril and valsartan would confer greater renoprotection than single therapy alone in this diabetic rat.

Subjects and methods

Research design

Six-week-old female heterozygous transgenic (mRen-2)27 rats (147 ± 6 g) were either made diabetic by a single tail-vein injection of STZ (Sigma, St Louis, MO, USA; 55 mg/kg diluted in 0.1 mol/L citrate buffer, pH 4.5), or received a control vehicle (non-diabetic) of citrate buffer, following an overnight fast, as previously described [3,4]. Female heterozygous Ren-2 rats were used because they do not require anti-hypertensive treatment for survival. Non-diabetic and diabetic rats were then randomized to receive either no treatment (non-diabetic, n = 6; diabetic, n = 10), the AT$_1$ receptor antagonist valsartan (CIBA Geigy Ltd, Basel, Switzerland) (non-diabetic, n = 10; diabetic, n = 7), the ACEI perindopril (Servier Laboratories, Neville, France) (non-diabetic, n = 6; diabetic, n = 6) or a combination of valsartan and perindopril (non-diabetic, n = 10; diabetic, n = 9). These numbers of rats were used in the evaluation of all the described parameters, except for glomerulosclerotic index and kidney cortical collagen staining where six rats/group were studied. Mono- or combined therapy commenced from 2 days post-STZ or control vehicle and continued for a period of 12 weeks.

All rats were allowed free access to tap water and standard rat chow (GR2, Clark-King & Co., Gladysville, NSW, Australia). Each week, rats were weighed and blood glucose (non-diabetic, 6 ± 1 mmol/L; diabetic, 27 ± 3 mmol/L; mean ± SEM) was estimated using an Ames glucometer (Elkhart USA). Diabetic animals received a daily injection of insulin (4–6 units i.p., Ultratrad, Novo Nordisk, Denmark) to promote weight gain and to reduce mortality. At 4, 8 and 12 weeks post-STZ or control vehicle, Ren-2 rats were housed in metabolic cages to measure 24 h fluid intake and urinary output as well as collecting urine samples for subsequent estimations of albumin excretion rate. All experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia’s Code for the Care and Use of Animals for Scientific Purposes.

Blood pressure

Systolic blood pressure (SBP) was recorded in conscious rats by tail cuff plethysmography at the same time of day (model PE-300 programmed electrophysymomanometer; Narco Biosystems, Inc., Texas, USA). The aim of mono- and combined therapy was to administer doses that maintained normotension (approximately 110–120 mmHg). Parallel studies in our laboratory have shown that perindopril administered in drinking water at a dose of 6 mg/kg/day normalizes SBP in non-diabetic and diabetic Ren-2 rats [3]. This perindopril monotherapy regimen was used again in the present study. For valsartan monotherapy, valsartan did not dissolve in drinking water and therefore was administered by gavage. Over the first 2 weeks of treatment, valsartan monotherapy administered at 20 mg/kg/day by gavage consistently normalized SBP; therefore treatment was continued at this dose for the remainder of the 12-week experimental period. For the combined therapy we sought accurate dosing of RAS blockers; hence both perindopril and valsartan were administered by gavage. It was anticipated that in combination therapy with valsartan (20 mg/kg/day, gavage) lower doses of perindopril would be necessary to prevent hypotension.

Using a combined regimen of 20 mg/kg/day of valsartan and 2 mg/kg/day of perindopril, all rats were hypotensive within 2 days of treatment (Figure 1). The doses of each drug were therefore progressively lowered over the next 2 weeks, resulting in a treatment regimen that led to SBP levels within the normotensive range (3 mg/kg/day valsartan and 0.5 mg/kg/day perindopril) (Figure 1). These doses of valsartan and perindopril were then used for the combined therapy regimen for the 12 week study in non-diabetic and diabetic Ren-2 rats. Having established these mono- and combined therapy regimens, SBP was measured at least twice weekly in the first 4 weeks of the study and then again at 8 and 12 weeks post-STZ diabetes or control vehicle.

Fig. 1. Systolic blood pressure in STZ diabetic heterozygous transgenic (mRen-2)27 rats treated with varying doses of a combination of valsartan and perindopril. V, valsartan; P, perindopril. Values are mean ± SEM. n = 9 rats/group. *P < 0.05 between all groups.
Renal function

Glomerular filtration rate (GFR) was determined 1–2 days prior to sacrifice by a single injection isotopic method [3]. In brief, the isotope $^{99m}$Tc-diethylene triamine penta-acetic acid was injected into the tail vein and blood sampled after 43 min. Plasma radioactivity was measured and compared with a reference prepared at the time of injection. GFR was calculated as clearance $= V \times \ln(P_o/P_t)/t$, where $V$ is the volume of distribution, $P_o$ the plasma concentration at injection, and $P_t$ the observed plasma concentration at $t$ min after injection. Urinary albumin concentration was determined using a double antibody radioimmunoassay [3,4].

Kidney histopathology

At 12 weeks post-STZ or control vehicle, Ren-2 rats were anaesthetised with pentobarbitone sodium (Nembutal, 50 mg/kg body weight i.p., Boehringer Ingelheim, Australia) and perfused via the abdominal aorta with 0.1 mol/l phosphate-buffered saline (approximately 150 ml, pH 7.4, 180–220 mmHg) for 1–2 min to remove circulating blood. The right kidney was then perfusion fixed for 5 min with 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4 [3,4]. The kidney was then removed, sliced transversely, and post-fixed overnight. After routine processing through graded alcohols, the kidneys were embedded in paraffin and sectioned at 3 μm [3,4].

Overall changes in kidney structure were observed in at least three randomly selected sections from each kidney. Sections were stained with either H&E to examine cell structure, periodic acid-Schiff (PAS) reagent to identify changes in basement membranes and glycogen deposition, and Masson’s modified trichrome to demonstrate collagen matrix [3,4]. Glomerulosclerosis was quantitated in a double-masked manner on 3-μm paraffin sections stained with PAS reagent [3,4]. Between 150 and 200 randomly chosen glomeruli from each rat kidney were graded for sclerosis. Glomeruli were graded carefully in a sequential manner so as not to grade the same glomerulus twice. Glomerulosclerosis was defined as glomerular basement membrane thickening, mesangial hypertrophy, and capillary occlusion. The degree of sclerosis in each glomerulus was subjectively graded on a scale of 0–4: grade 0, normal; grade 1, sclerotic area up to 25% (minimal); grade 2, sclerotic area 25–50% (moderate); grade 3, sclerotic area 50–75% and grade 4, sclerotic area 75–100% (severe). A glomerulosclerotic index (GSI) was then calculated using the formula:

$$GSI = \sum_{i=0}^{4} F(i)$$

where $F(i)$ is the percentage of glomeruli in the rat with a given score of $(i)$.

Kidney cortical collagen was evaluated in at least four randomly chosen sections from each rat kidney ($n = 6$) that were stained with Masson’s trichrome. Bright field images were captured and digitized using a Fujix HC-2000 digital camera (Fujif, Tokyo, Japan). Collagen staining was quantitatively measured to determine the proportion of each area occupied by blue stain using computerized image analysis (Analytical Imaging Station, Imaging Research, Australia).

Statistics

Comparisons of normally distributed variables between non-diabetic and diabetic groups were analysed by a one-way analysis of variance (ANOVA) followed by a Fisher’s individual post hoc comparison. All statistics were performed using Microsoft Minitab version 10.5. A change was considered statistically significant if $P<0.05$. All values are expressed as mean±standard error of the mean (SEM), except for albuminuria, which are shown as geometric means ×/± tolerance factors. The minimum number of animals required for statistical significance is six rats per group, which was estimated using an $z$ value of 0.05 and a $\beta$ value of 0.1.

Results

Blood pressure

There was no significant difference in SBP between untreated non-diabetic and diabetic Ren-2 rats (Figure 2). Throughout the 12-week experimental period, SBP was significantly lower in treated non-diabetic and diabetic Ren-2 rats compared with untreated animals (Figure 2). The perindopril-treated non-diabetic and diabetic groups had a significant fall in SBP when compared to untreated rats ($P<0.01$), but SBP was still significantly higher than groups that received valsartan either as monotherapy or in combination with perindopril ($P<0.05$).

**Fig. 2.** Systolic blood pressure in non-diabetic (panel A) and STZ diabetic (panel B) heterozygous transgenic (mRen-2)27 rats treated with mono- and combination therapies of valsartan and perindopril. V, valsartan; P, perindopril. [No treatment]; [20 mg/kg/day valsartan]; [6 mg/kg/day perindopril]; and [a], a combination of 3 mg/kg/day valsartan plus 0.5 mg/kg/day of perindopril. Values are means±SEM. $n=6$–10 rats group. *$P<0.05$ vs untreated; **$P<0.01$ vs untreated; †$P<0.05$ vs valsartan and valsartan plus perindopril.
Table 1. Body weight, glomerular filtration rate, glomerulosclerotic index, and percentage of severe glomerulosclerosis in non-diabetic and STZ diabetic heterozygous transgenic (mRen-2)27 rats

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>GFR (ml/min)</th>
<th>GSI</th>
<th>Grades 3 &amp; 4, severe GS (%)</th>
<th>Cortical collagen staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic control</td>
<td>281 ± 4</td>
<td>3.5 ± 0.2</td>
<td>0.64 ± 0.1</td>
<td>3 ± 1</td>
<td>0.12 ± 0.08</td>
</tr>
<tr>
<td>Diabetic</td>
<td>219 ± 3*</td>
<td>1.6 ± 0.1*</td>
<td>1.74 ± 0.1*</td>
<td>27 ± 3*</td>
<td>21.2 ± 1.13*</td>
</tr>
<tr>
<td>Non-diabetic + V</td>
<td>305 ± 8.2</td>
<td>2.8 ± 0.1</td>
<td>0.25 ± 0.1</td>
<td>0</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Diabetic + V</td>
<td>249 ± 7.1</td>
<td>2.9 ± 0.3</td>
<td>0.89 ± 0.2**</td>
<td>16 ± 3*</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Non-diabetic + P</td>
<td>308 ± 5</td>
<td>2.9 ± 0.2</td>
<td>0.39 ± 0.1</td>
<td>0</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Diabetic + P</td>
<td>268 ± 8</td>
<td>2.7 ± 0.2</td>
<td>0.71 ± 0.1**</td>
<td>11 ± 1*</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Non-diabetic + V + P</td>
<td>266 ± 19.2</td>
<td>2.9 ± 0.2</td>
<td>0.32 ± 0.1</td>
<td>0</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Diabetic + V + P</td>
<td>244 ± 13.6</td>
<td>2.5 ± 0.2</td>
<td>0.50 ± 0.1**</td>
<td>0#</td>
<td>&lt;0.01%</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. n = 6 to 10 rats per group. GFR, glomerular filtration rate; GSI, glomerulosclerotic index; GS, glomerulosclerosis; V, valsartan; P, perindopril. *P < 0.01 compared to all groups; **P < 0.05 compared to respective treated non-diabetic; †P < 0.01 compared to all non-diabetic groups and diabetic + V + P; ‡P < 0.05 compared to untreated diabetic; #P < 0.05 compared to diabetic + P and diabetic + V.

Animal survival, body weight, glomerular filtration rate, and albuminuria

There were no deaths in non-diabetic animals and one death in each of the untreated diabetic and diabetic + valsartan groups. Body weight was reduced with diabetes and improved with mono- and combination therapy (Table 1). The decline in GFR associated with diabetes was largely prevented by valsartan, perindopril, and the combination of valsartan and perindopril (Table 1). In non-diabetic rats, treatment did not alter GFR when compared to untreated animals. Urinary albumin excretion was increased in diabetic rats, and was reduced to a similar extent with valsartan, perindopril, and the combination of valsartan and perindopril (Figure 3). Treatment did not influence urinary albumin excretion in non-diabetic rats.

Histopathology

In untreated non-diabetic rats, the kidney cortex appeared normal with only some glomeruli displaying thickened glomerular basement membranes (GBM) (Figure 4A). In contrast, in untreated diabetic rats most glomeruli exhibited thickened GBM, capillary occlusion, and mesangial expansion (Figure 4B). In addition, many cortical tubules were vacuolated (Figure 4B). In non-diabetic and diabetic rats treated with either valsartan (Figures 4C and 4D), perindopril (not shown), or a combination of valsartan and perindopril (Figures 4E and 4F), glomerular pathology was improved, although in diabetic kidney cortex some tubules remained vacuolated.

Diabetes was associated with an increase in GSI (Table 1). Compared to untreated diabetic rats, GSI was lower in diabetic rats treated with valsartan and perindopril monotherapy and the combination of valsartan and perindopril. Only in diabetic Ren-2 rats treated with combination therapy were no sclerosed glomeruli present (Table 1). Both monotherapy and combined therapy lowered GSI in non-diabetic rats compared with untreated animals.

In the tubulointerstitium of both the kidney cortex and medulla, increased collagen and inflammatory cells were observed in diabetic Ren-2 rats compared to non-diabetic controls. All treatments reduced tubulointerstitial injury to a similar extent (Table 1).

Discussion

The present study reports that in a model of severe renal pathology in the diabetic Ren-2 rat, low-dose combination therapy (valsartan plus perindopril) was more efficacious than either monotherapy in improving severe diabetic glomerulosclerosis and equally as effective at ameliorating the decline in renal function and increase in albuminuria. With respect to blood pressure, all treatments were antihypertensive; however, perindopril was less effective than valsartan monotherapy despite affording similar renoprotection. However, one cannot exclude that there is a degree of dissociation between blood pressure and renal injury in the diabetic Ren-2 rat model [4], and that possible differences in pharmacokinetics and pharmacodynamics between the drugs also play an important role.
A similarity in the renoprotective effects of AT-RB and ACEI has been reported in a variety of renal pathologies [12,13]. In all cases, either agent has been shown to reduce proteinuria and glomerulosclerosis to a similar degree. The few studies that have compared monotherapy vs combined AT-RB and ACEI have reported no additional benefit of combined therapy on renal structure and function in the 5/6 renal mass ablation model [14], cyclosporin-induced interstitial fibrosis [15], and uninephrectomized STZ diabetic spontaneously hypertensive rats (SHR) [16]. With regard to the attenuation of the decline in renal function, our findings are in agreement with these studies; combined valsartan and perindopril treatment reduced
albuminuria and attenuated the decline in GFR to similar extents as monotherapy. However, although fewer numbers of rats were studied for renal structural damage, a combination of valsartan and perindopril exerted an additive effect in reducing the development of severe glomerulosclerosis. Indeed, unlike STZ diabetic Ren-2 rats treated with monotherapy, severely sclerosed glomeruli, consisting of a hyalinized renal corpuscle and extreme basement membrane thickening, were never observed in diabetic rats treated with combination therapy. An additive protective effect in the heart has also been reported in the SHR and transgenic Ren-2 rat, with combined low dose AT-RB (losartan) and ACEI (enalapril) treatment inducing a greater reduction in left ventricular weight/body weight ratio than either treatment alone [17,18]. The present study was performed in a genetic strain, the transgenic Ren-2 rat, which overexpresses various components of the RAS, including renin, in response to chronic hyperglycaemia [3,4]. This phenomenon may not occur in all rat strains and therefore one must be cautious in extrapolating these findings firstly to other experimental contexts and secondly to humans.

In many experiments designed to compare the effects of monotherapy vs combined AT-RB and ACEI on the progression of renal pathology, the high doses used for monotherapy were added together for the combined treatment protocol [14–16]. Unlike the high-dose approach, we found that a few days of the combined treatment elicited hypotension, suggesting increased sensitivity of the Ren-2 rat to dual blockade of the RAS. To achieve and maintain normotension, valsartan was progressively reduced from 20 to 3 mg/kg/day and perindopril from 6 to 0.3 mg/kg/day. An additive effect of AT-RB and ACEI on blood-pressure reduction has been previously reported in the transgenic Ren-2 rat [18], and most probably relates to this being a model of renin-dependent hypertension and organ pathology [19].

The added renoprotective effects afforded by combined RAS blockade in this model adds further support to the primary involvement of a tissue-based RAS rather than blood pressure or the circulating RAS in the development of diabetic nephropathy. A number of studies suggest an enhanced kidney RAS may induce local cellular injury [2–4]. We have previously shown that the glomerular and tubular lesion in the STZ diabetic Ren-2 rat is associated with an increase in both juxtaglomerular and proximal tubular renin, suggesting a pathological role for the RAS at these sites [3]. In the diabetic Ren-2 rat model both RAS blockade and combined endothelin type A and B receptor antagonism were equally antihypertensive; however, only RAS blockade improved renal structure and function, indicating that the local RAS confers renoprotection via a blood-pressure-independent mechanism [4]. Despite monotherapy and low-dose combined therapy similarly reducing blood pressure, severe glomerulosclerosis was only completely prevented in the rats receiving combined treatment. The mechanisms by which the diabetic state induces overexpression of the kidney RAS are unknown. It has been reported that elevated glucose per se can sensitize kidney cells to the actions of Ang II [20], increasing the production of proinflammatory cytokines such as transforming growth factor β1 [21] which are also Ang II dependent and may directly increase angiotensinogen gene expression and protein synthesis [22].

With respect to clinical studies, there is increasing evidence of a similarity in renal protection between ACEI and AT-RB [23]. Recently it has been shown in type 1 diabetic patients that the ACEI enalapril and the angiotensin type 1 receptor antagonist losartan, had similar effects on albuminuria in this population [24]. Combination of ACEI and AT-RB has been very well studied. In normotensive volunteers maintained in mild sodium depletion, an additive effect of ACEI and angiotensin type 1 receptor antagonism on blood pressure has been described [25]. A benefit of this combination in heart failure has also been demonstrated [26]. In renal diseases, two small studies have described different renal effects of combination ACEI and AT-RB therapy [27,28]. In a study of type II diabetic patients, 1 week of combination treatment had no effect on proteinuria or blood pressure [27]. By contrast, in a 4-week study of patients with IgA nephropathy, a combination of ACEI and losartan reduced proteinuria [28]. The recently completed CALM study comparing the effects of the combination of the angiotensin type 1 receptor antagonist candesartan and the ACEI lisinopril documented significant increased reductions in blood pressure with combination treatment and a substantial trend toward further reductions in albuminuria [29].

In conclusion, combination therapy of an AT1 receptor blocker and ACEI appears to be a potent approach for effectively decreased blood pressure and preventing the development of severe glomerulosclerosis in this model of accelerated diabetic nephropathy. These findings provide a rationale for applying such combination regimens in the prevention of severe renal pathologies such as diabetic nephropathy.

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Combined AT1 and ACE blockade in diabetic nephropathy


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