Functional and Partial Morphological Regression of Established Renal Injury in the Obese Zucker Rat by Blockade of the Renin-Angiotensin System

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Key Words

Obese Zucker rat • Glomerulosclerosis • Tubulointerstitial fibrosis • Glomerular cell number • Proteinuria • Angiotensin-converting enzyme inhibitors • Angiotensin II receptor 1 blockers

Abstract

Background/Aims: In experimental nephropathies, inhibitors of the renin-angiotensin-system (RAS) halted the progression, or even induced a regression in renal injury. We studied the potential of the angiotensin-converting enzyme (ACE) inhibitor perindopril and the angiotensin receptor blocker candesartan to reverse the established renal injury in the obese Zucker rat (OZR). **Methods:** Forty 4-week-old OZR were uninephrectomized and fed a high-protein diet. After 16 weeks, they were randomized into 4 groups (n = 10 each) with comparable proteinuria: (1) control group sacrificed immediately for baseline data, and groups gavaged daily for 8 weeks with (2) placebo, (3) perindopril (1 mg/kg/day), or (4) candesartan (10 mg/kg/day). **Results:** Both drugs reduced systolic blood pressure (perindopril –16%, p < 0.001; candesartan –10%, p < 0.05), renal hypertrophy, and proteinuria (perindopril to 32%; candesartan to 37% of pretreatment values). Glomerulosclerosis was halted (perindopril p < 0.001; candesartan p < 0.05), and the numbers of glo-

merular endothelial and podocyte cells were restored. Mesangiolysis was reversed by perindopril. Metabolic and oxidative parameters were either stabilized (perindopril), or improved (candesartan). **Conclusion:** In the OZR late inhibition of RAS halts the progression of glomerulosclerosis, reverses mesangiolysis and prevents the decline in glomerular endothelial cell and podocyte numbers. Tubulointerstitial fibrosis and vascular injury remain unchanged. Proteinuria shows marked regression.

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Introduction

Nephropathy due to type 2 diabetes mellitus is the leading cause of end-stage renal disease. A key factor in the progression of diabetic and non-diabetic renal disease is the renin-angiotensin system (RAS). In humans, or rodent models, angiotensin-converting enzyme (ACE) inhibitors (ACEIs) and angiotensin II (AngII) type 1 receptor blockers (ARBs) are effective in the prevention and control of renal damage. In experimental settings inhibitors of RAS even reversed the established non-diabetic renal injury [1–3], while pancreatic transplantation in humans reversed diabetic nephropathy (DN) [4].

Fig. 1. The experimental protocol.

Obese Zucker rats (OZR, a model of type 2 diabetes mellitus), develop hyperphagia, obesity, hyperglycemia, hyperinsulinemia, hyperlipidemia, hyperaggregation of platelets, moderate hypertension, and (at approximately 40 weeks of age) renal damage [5]. The effects of ACEIs, ARB and their combination in preventing renal injury in OZR have already been described [6], but not evaluated in established DN. We show that 8-week treatment with the ACEI perindopril and the ARB candesartan, initiated in established DN, ameliorates and even reverses functional and several aspects of glomerular kidney damage.

Materials and Methods

Animals

The research protocol was approved by the Ethics Committee for Animals (Bratislava, Slovakia). Forty 1-month-old male OZR (Iffa Credo, Lyon, France) were subjected to unilateral nephrectomy under intraperitoneal ketamin/xylazin anesthesia [7]. Animals were housed under standard conditions with free access to water. After uninephrectomy, rats were switched from standard chow to a high-protein diet (50%, Altromin, Lage, Germany). Four months later they were randomized into 4 groups (10 animals/group) with comparable proteinuria (fig. 1). Control rats were sacrificed before any treatment was initiated. By gavage, placebo rats received a 1% water solution of hydroxyethyl cellulose (HEC) in a volume of 5 ml/kg. Perindopril (Servier, Paris, France) was administered at a dose of 1 mg/kg/day, and candesartan cilexetil (Astra Zeneca, Wedel, Germany) at a dose of 10 mg/kg/day, dissolved in 1% HEC, 7 days/week for 8 weeks.

Rats were weighed weekly. Before sacrifice, systolic blood pressure (SBP) was measured by tail phetysmography. Twenty-four-hour urine was collected in metabolic cages before randomization and sacrifice. Blood was sampled under anesthesia from the abdominal aorta.

Biochemical Analyses

Routine blood and urine biochemistry (Vitros 250 Analyzer; J&J, Rochester, Minn., USA) and blood count (Sysmex K-21 Ana-

lyzer; Kobe, Japan) were analyzed. In plasma, the immunoreactive insulin concentration (Linco Research, St. Charles, Mo., USA) and advanced oxidation protein products (AOPPs) [8], and in erythrocytes glutathione peroxidase (GPX) activity (Randox Laboratories, Crumlin, UK) were determined.

Renal Histology

After retrograde perfusion fixation with glutaraldehyde via the abdominal aorta, kidneys were weighed and dissected into 1-mm-wide slices in a plane perpendicular to the interpolar axis [7]. Ten pieces/kidney were embedded in Epon-Araldite. Semithin (1- μ m) slices were stained with methylene blue and basic fuchsin. Four-micrometer sections embedded in paraffin were stained with hematoxylin/eosin (HE) and periodic acid-Schiff (PAS). All morphological evaluations were performed in a blinded manner.

Morphological Investigations

Semiquantitative

Mesangiolytic (a hallmark of early glomerular damage in DN) was determined utilizing a 0–4 scoring system (0 = normal to 4 = severe damage) in 100 glomeruli/animal [7].

The degree of mesangial matrix expansion and sclerosis within the glomerular tuft (an index of progression) was determined on PAS-stained sections using a semiquantitative scoring system from 0 to 4 (0 = normal glomerulus, 4 = severe damage) in 100 glomeruli/animal [7, 9].

Tubulointerstitial and vascular damage indices were assessed on PAS-stained paraffin sections using the previously described scoring system [7, 9].

Glomerular Geometry

The volume density of glomeruli and the area density of glomerular tufts were measured by point counting on HE sections [7, 9]. The total number of glomeruli was derived from the total volume of the renal cortex and the number of glomeruli per cortex volume. The mean glomerular tuft volume was determined.

Semithin sections were qualitatively inspected for glomerular cellular changes (podocyte hypertrophy and degeneration, and mesangial or endothelial cell hyperplasia). Five sections/animal (at least 30 glomeruli) were analyzed quantitatively for glomerular cell number and volume determination [9].

Table 1. Animal data, blood and urine chemistry

	CTRL	PLAC	PER	CAN	ANOVA	
					F	p
Body weight t0, g	524 ± 32	542 ± 52	526 ± 41	508 ± 40	1.57	0.223
Body weight t8, g	ND	543 ± 64	493 ± 62	476 ± 52	3.26	0.055
Kidney weight, g	2.2 ± 0.3	3.4 ± 0.9 ⁺⁺	2.6 ± 0.5*	2.3 ± 0.3***	6.63	0.005
Kidney/body weight, mg/g	4.3 ± 0.5	6.1 ± 1.2 ⁺⁺	5.2 ± 1.0	4.9 ± 0.2*	4.50	0.021
SBP, mm Hg	ND	147 ± 17	124 ± 12***	133 ± 11*	7.10	0.004
Creatinine, μmol/l	53 ± 24	64 ± 16	74 ± 13	74 ± 17	1.28	0.295
Cl _{crea} , ml/min	0.9 ± 0.4	0.8 ± 0.2	0.6 ± 0.1	0.7 ± 0.2	1.82	0.184
Glucose, mmol/l	28 ± 9	23 ± 3	24 ± 5	24 ± 7	0.13	0.878
IRI, ng/ml	1.0 ± 0.9	2.2 ± 1.0 ⁺	2.1 ± 0.8	1.3 ± 0.4*	3.60	0.042
Cholesterol, mmol/l	7.3 ± 2.0	6.7 ± 1.4	6.5 ± 1.0	6.2 ± 1.1	0.45	0.645
Triglycerides, mmol/l	2.2 ± 0.4	2.1 ± 0.7	2.6 ± 0.9	1.3 ± 0.7	6.58	0.005
Leukocyte count, × 10 ⁹ /μl	4.6 ± 1.4	8.8 ± 5.2 ⁺	5.2 ± 2.8	6.8 ± 4.5	1.73	0.198
AOPPs/Alb, μmol/g	2.2 ± 0.6	1.7 ± 0.6	1.6 ± 0.5	0.8 ± 0.6***	7.28	0.003
GPX activity, U/g Hb	29.5 ± 5.7	25.0 ± 3.1 ⁺	28.5 ± 4.0	28.5 ± 4.3	2.43	0.109

CTRL = Control obese Zucker rats sacrificed before initiation of the treatment; PLAC = placebo; PER = perindopril; CAN = candesartan; t0 = before initiation of treatment; t8 = after 8 weeks of treatment; SBP = systolic blood pressure; Cl_{crea} = creatinine clearance; IRI = immunoreactive insulin; AOPPs = advanced oxida-

tion protein products; Alb = albumin; GPX = glutathione peroxidase; Hb = hemoglobin; ND = not determined (technical reasons).

⁺ p < 0.05, ⁺⁺ p < 0.01 vs. CTRL; * p < 0.05, *** p < 0.001 vs. PLAC.

Statistics

Data are expressed as mean ± SD. Control and placebo groups were compared employing Student's t test or the Mann-Whitney U test, where appropriate. One-way ANOVA with Dunnett's post-hoc test was used to compare placebo and treated groups. Two-way ANOVA with Duncan's post-hoc test was used to analyze the data on diuresis and proteinuria. A p value of <0.05 was considered significant.

Results

Body Weight, Kidney Weight and SBP (table 1)

During treatment, the body weight of the placebo rats remained stable, while it tended to decrease in the treated groups. Compared to placebo, perindopril- and candesartan-treated rats had lower SBP by 16% (−23 mm Hg; p < 0.001) and 10% (−14 mm Hg; p < 0.05), respectively. Kidney weight was higher in placebo rats (+55%, p < 0.01 vs. controls). Both treatments effectively halted hypertrophy, but after correction for body weight it remained significant only in the candesartan group (vs. placebo, p < 0.05).

Renal Function (table 1)

An insignificant rise in mean plasma creatinine (21%), and a decreased creatinine clearance (−12%; n.s.) were observed in placebo rats (vs. controls). Both treated groups had an insignificantly higher plasma creatinine concentration (+16% vs. placebo), and lower creatinine clearance (perindopril −33%; candesartan −14%; n.s.).

At treatment initiation, groups had comparable diuresis and proteinuria (fig. 2, 3). After treatment, diuresis increased. In the placebo group proteinuria increased (+21%; n.s.), perindopril and candesartan lowered proteinuria to 32 (p < 0.01) and 37% (p < 0.001) of pretreatment values, respectively.

Kidney Morphology (fig. 4, 5; table 2)

DN in placebo rats markedly progressed during the treatment period: mesangiolysis (+100%); glomerulosclerosis (+33%); tubulointerstitial fibrosis (+56%), and vascular damage scores (+61%) increased (all p < 0.001). Candesartan stopped the progression of mesangiolysis, while perindopril reversed it (−32%). Both treatments halted the progression of glomerulosclerosis. Mild tubulointerstitial or vascular damage remained unaffected.

Placebo rats developed glomerular hypertrophy; perindopril slightly ameliorated it (−11% vs. placebo; n.s.).

Fig. 2. Time course of diuresis. CTRL = Controls; PLAC = placebo; PER = perindopril; CAN = candesartan; t0 = before the treatment; t8 = after 8 weeks of treatment.

Fig. 3. Time course of proteinuria. CTRL = Controls; PLAC = placebo; PER = perindopril; CAN = candesartan; t0 = before the treatment; t8 = after 8 weeks of treatment. *** $p < 0.001$ vs. PLAC.

The total number of glomerular cells did not differ significantly between placebo and control rats. In both treatment groups endothelial cell and podocyte numbers were significantly higher than in the placebo group. In parallel, with a trend towards lower endothelial cell number, glomerular capillary length density (as a three-dimensional parameter of glomerular capillarization) was significantly lower in the placebo group vs. the controls. Both treatments had no significant effect on glomerular capillarization.

Blood Chemistry (table 1)

All rats were hyperglycemic. Placebo rats had higher insulinemia than the controls, or candesartan-administered animals. All groups showed dyslipidemia, which was not significantly reduced by the treatments. Placebo rats had significantly higher leukocyte count (vs. controls), which was insignificantly reduced by both treatments.

Oxidative Status (table 1)

Plasma AOPPs did not differ significantly between the control and placebo groups. Candesartan reduced AOPP levels (−38% vs. placebo; $p < 0.01$). GPX activity was significantly lower in the placebo rats (vs. the controls), and both treatments only partially restored it.

Discussion

To date, no data are available on the secondary prevention of renal injury in the OZR by blockade of the RAS. The major finding of this study shows that the RAS blockade in established DN in OZR, either with perindopril

Fig. 4. Renal damage scores. CTRL = Controls; PLAC = placebo; PER = perindopril; CAN = candesartan; t0 = before the treatment; t8 = after 8 weeks of treatment; MGI = Mesangiolysis index; GSI = glomerulosclerosis index. * $p < 0.05$, *** $p < 0.001$ vs. PLAC; ++ $p < 0.01$ vs. CTRL.

or candesartan, not only halts the progression of several markers of renal injury, but even induces a partial regression of glomerular damage. Both treatments retarded the progression of glomerulosclerosis and preserved glomerular cells (podocytes, endothelial cells, glomerular capillary supply). Candesartan halted and perindopril induced a limited regression of mesangiolysis (in contrast to other studies) [6]. Tubulointerstitial and vascular sclerosis scores were not significantly affected. Both treatments suppressed proteinuria. The missing effect on the extra-glomerular structures may reflect the persisting risk factors of renal damage, e.g. hyperglycemia, hypertension, dyslipidemia, obesity, and high protein intake. Moreover, in the OZR the systemic RAS is downregulated, limiting the effect of ACE inhibition/blockade [10].

Fig. 5. Representative changes in glomerular morphology. Paraffin sections. **A** Glomerulus of a uninephrectomized OZR after a 4-month-long period without treatment (basal data). **B** Glomerulus from placebo-administered OZR showing irregular structures

of the capillary tuft with capillary widening, capillary dilatation and dissolution of the mesangium (mesangiolysis score 1–2). **C, D** Glomerulus of perindopril- (**C**) and candesartan- (**D**) treated OZR with a nearly normal capillary structure and mesangium.

A major mechanism of renoprotection under RAS blockade is the antihypertensive effect, as in our study. However, the decline in blood pressure seems to be too mild to explain the marked improvement in renal injury, thus other mechanisms have to be considered.

Oxidative Stress

AngII promotes generation of reactive oxygen species (ROS) which worsen insulin resistance, diabetes, and its complications [11]. AOPPs reflect, and trigger, an oxidative burst of phagocytes [8]. In our study, candesartan lowered AOPP levels, similar to peritoneal dialysis patients [12]. In line with the increased AOPPs, the placebo

rats had a higher leukocyte count which, even within the normal range, is associated with macro- and microvascular complications in diabetics [13]. While perindopril increased the activities of antioxidant enzymes in hypertensive patients [14], decreased GPX activity in our placebo rats was only partially restored under both treatments. Thus, other antioxidative mechanisms could have been involved.

Carbohydrate/Lipid Metabolism

ARB administration decreases peripheral insulin resistance in type 2 diabetics and animal models [15]. We observed the insulin-sensitizing effects of candesartan,

Table 2. Renal histomorphology

	CTRL	PLAC	PER	CAN	ANOVA	
					F	p
TSI	0.14 ± 0.06	0.32 ± 0.08 ⁺⁺⁺	0.28 ± 0.05	0.25 ± 0.07	2.60	0.094
VSI	0.11 ± 0.03	0.28 ± 0.07 ⁺⁺⁺	0.33 ± 0.07	0.28 ± 0.09	1.38	0.269
Glomerular volume, $\mu\text{m}^3 \cdot 10^3$	629 ± 36	705 ± 50 ⁺⁺	649 ± 24	688 ± 55	3.39	0.050
Length density of glomerular capillaries, mm/mm ³	8,599 ± 509	7,543 ± 548 ⁺⁺⁺	7,319 ± 882	8,181 ± 684	3.52	0.046
Podocyte number	110 ± 23	98 ± 16	129 ± 21 ^{***}	121 ± 16*	7.63	0.003
Mesangial cell number	215 ± 36	249 ± 50	244 ± 52	273 ± 52	0.823	0.451
Endothelial cell number	380 ± 51	333 ± 47	410 ± 63 ^{***}	443 ± 48 ^{***}	10.89	0.001
Parietal cell number	199 ± 22	218 ± 21	226 ± 13	230 ± 36	0.55	0.582
Total number of glomerular cells	904 ± 73	898 ± 99	1,009 ± 128	1,067 ± 102 ^{***}	5.84	0.008

CTRL = Control obese Zucker rats sacrificed before initiation of treatment; PLAC = placebo; PER = perindopril; CAN = candesartan; TSI = index of tubulointerstitial damage; VSI = index of vascular damage.

⁺⁺ p < 0.01, ⁺⁺⁺ p < 0.001 vs. CTRL; * p < 0.05, ^{***} p < 0.001 vs. PLAC.

similar to a study in OZR rats administered irbesartan [16]. Neither treatment influenced dyslipidemia, despite the reduction in proteinuria. In other studies candesartan either lowered or induced mild changes in plasma lipids [17, 18]. Although perindopril did not significantly affect metabolic parameters, in hypertensive type 2 diabetics it improved some metabolic parameters compared with candesartan [18].

Fat Tissue

Obesity is a risk factor for progression of renal disease [19]. Adipocytes produce all components of the RAS [20]. RAS blockade in rodents lowered body weight gain, fat mass, adipocyte size and AT₁ receptor number [21]. Thus, the decline in body weight in both treatment groups could be another protective mechanism of RAS blockade. However, in clinical studies, lowering of body weight under RAS blockade has not been reported.

Renal Mechanisms

In line with previous reports [3, 22, 23], preservation of podocytes, glomerular endothelial cells, and reduced mesangial sclerosis were important renoprotective effects of RAS blockade also in established DN in OZR rats. As vascular endothelial growth factor (VEGF) is considered a key downstream mechanism, the preservation of endothelial cells and podocytes could have been mediated via VEGF. Perindopril resulted in higher capillary density and VEGF synthesis in the myocardium of OZR rats [24], and in the ischemic hindlimb of diabetic mice [25].

The lowered glomerular filtration rate in the treated groups is most probably functional, partly via decreased systemic blood pressure, as is well known after ACEI administration [26].

Since single ACEI and ARB compounds differ in their pharmacological properties, we cannot conclude whether our data represent a class effect or merely that of the drugs employed. It is unclear whether RAS blockade could reverse DN also in humans.

In conclusion, the late intervention with perindopril and candesartan in established DN in uninephrectomized OZR rats halted the progression and even induced a partial regression in glomerular injury, preserving glomerular cells and capillaries. The blood pressure-lowering actions of RAS blockade as well as additional effects (reduction of oxidative stress, improved carbohydrate metabolism) are to be considered, in particular for candesartan.

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