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Anne-Roos S Frenay¹, Willem-Peter T Ruifrok², Marian Bulthuis¹, Sippie Huitema¹, Rudolf A de Boer² and Harry van Goor¹

Abstract

Introduction: Erythropoietin (EPO) has cytoprotective and angiogenic properties and has a beneficial effect in ischaemic conditions. Since the development of renal interstitial abnormalities are often associated with ischaemia, we studied the effects of the long-acting EPO analogue darbepoetin alpha (DA) on kidney damage in TGR(mRen2)27 (Ren2) rats.

Materials and methods: Ren2 rats were randomised to DA or vehicle (VEH) or to DA + angiotensin converting enzyme inhibitor (ACEi) or VEH + ACEi. Sprague Dawley (SD) rats served as controls. Blood pressure was measured weekly and 24-h urine was collected to measure proteinuria. Blood samples were collected for creatinine and haematocrit. Kidneys were studied for inflammation and pre-fibrosis. Renal mRNA expression was studied for EPO, EPO-receptor, collagen-3 α 1 and kidney injury molecule-1 (KIM-1).

Results: DA had no effect on SBP, serum creatinine and proteinuria. Interstitial and glomerular α -SMA expression was significantly increased in Ren2. ACEi but not DA improved the increased renal inflammatory and pro-fibrotic profile in Ren2 rats. DA on top of ACEi further reduced glomerular α -SMA and KIM-1 expression.

Conclusion: Long-term DA treatment has no beneficial effects on renal structural and functional changes in TGR(mRen2)27 rats in the time frame studied and the dose provided.

Keywords

ACEi, darbepoetin alpha, hypertension, kidney, renin–angiotensin system, Ren2

Introduction

Hypertension is present in approximately 85% of all chronic kidney disease (CKD) patients and can cause extensive vascular damage with subsequent interstitial and glomerular change.¹ TGR(mRen2)27 (Ren2), a monogenetic rat model, is used to study hypertension-related renal and cardiac disease. The mechanism of hypertension in this model is related to the activation of the renin–angiotensin system.² This ongoing systemic hypertension has an adverse effect on the vasodilated interstitial vascular compartment. This is accompanied by primary capillary injury and causes progressive obliteration of particular interstitial capillaries. This process then initiates chronic tubular ischemia ultimately leading to atrophy and ongoing interstitial damage.³

Erythropoietin (EPO) has multiple non-haematopoietic effects such as cytoprotection and anti-apoptosis and also plays an important role in the response to acute and chronic ischaemia and inflammation.^{4,5} Tissue protection by EPO after ischaemia and injury has been found in the brain, heart and kidney.^{5–8} In the kidney, administration of recombinant

human EPO at the time of ischaemic injury inhibits apoptosis and enhances tubular epithelial regeneration, thereby promoting renal functional recovery.^{6,9} These cytoprotective effects of EPO are caused by the binding of EPO to a heterodimeric complex, which exists of two EPO receptors and two beta common receptors (β CR). Binding to this receptor complex (EPOR2- β CR2) does not influence erythropoiesis.

We hypothesise that the long-acting EPO analogue darbepoetin alpha (DA) has beneficial effects on renal

¹Pathology and Medical Biology, University Medical Center Groningen and University of Groningen, The Netherlands

²Cardiology, University Medical Center Groningen and University of Groningen, The Netherlands

Corresponding author:

Anne-Roos S Frenay, Department of Pathology and Medical Biology, University Medical Center Groningen and University of Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.

Email: a.s.frenay@umcg.nl

structural and functional damage, induced by angiotensin II-mediated hypertension in rats with high renin.

Materials and methods

Animals

Experiments were conducted in 6-week-old male homozygous TGR(mRen2)27 rats. Age-matched SD rats served as controls. Ren2 rats and SD rats were purchased from the Max Delbrück Center for Molecular Medicine, Berlin–Buch, Berlin, Germany. All animals were housed under standard conditions at the animal research facility with free access to drinking solution and rat chow. All procedures were approved by the Committee for Animal Experiments of the University of Groningen.

Experimental design

Rats were randomised to different treatment groups. Ren2 rats received DA ($n = 14$) or vehicle (VEH) ($n = 13$) and the Ren2 controls received DA or VEH in combination with the ACE inhibitor Lisinopril (L6292; Sigma-Aldrich, St. Louis, MO, USA) (both $n = 5$). SD rats were also given DA or VEH ($n = 12$) and the SD controls received DA or VEH in combination with Lisinopril (both groups $n = 4$). After randomisation blood was collected for baseline values. For EPO administration the long-acting EPO analogue darbepoetin alpha (Aranesp, Amgen, Inc., Thousand Oaks, California, USA) was used. DA or VEH was administered by intraperitoneal injection at baseline, 3 and 6 weeks under full anaesthesia. DA was administered at a dose of 40 $\mu\text{g}/\text{kg}$ (equivalent to ~ 8000 units/kg) in 0.5 ml NaCl, the VEH group received 0.5 ml NaCl only. With every administration of DA or VEH, blood was sampled to determine haematocrit and creatinine. Lisinopril was dissolved in the drinking water and provided at a dose of 10 $\mu\text{g}/\text{ml}$. After 2 weeks of daily training, blood pressure was measured weekly in conscious animals using the tail-cuff method (Apollo 179; IITC Life Science, Woodland Hills, California, USA) as previously described.¹⁰ Body weight was determined weekly and 24-h urine was collected to measure proteinuria at baseline, 3 and 6 weeks. At sacrifice, 6 weeks after the start of the experiment, rats were anaesthetised. The aorta was cannulated and blood samples were taken. Coronal kidney slices were fixed with 4% paraformaldehyde for paraffin embedding or immediately snap frozen in liquid nitrogen and stored at -80°C for molecular analyses.

Qualitative real-time PCR

Rat renal tissue containing cortex and medulla was homogenised in lysis buffer and total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, USA).¹¹ RNA concentration was measured by nanodrop UV-detector (Nanodrop Technologies, Wilmington, Delaware, USA). cDNA was synthesised using Superscript II with Random

hexamer primers (Invitrogen). Gene expression levels were measured by qualitative real-time PCR (qPCR) (Applied Biosystems, Foster City, California, USA) based on the Taqman methodology. Primers were obtained from Applied Biosystems gene expression assays. The gene expression assays IDs were: EPO (Rn01481376_m1), EPO-receptor (Rn00566533_m1), Coll3- $\alpha 1$ (Rn01437683_m1), KIM-1 (Rn Rn00597703_m1), HPRT-F (GCC CTT GAC TAT AAT GAG CAC TTC A), HPRT-R (TCT TTT AGG CTT TGT ACT TGG CTT TT), HPRT-probe (ATT TGA ATC ATG TTT GTG TCA TCA GCG AAA GTG). The qPCR reaction mixture contained 20 ng cDNA template and 5 μl mastermix. Nuclease-free water was added to a total volume of 10 μl . Assays were performed in triplicate. The PCR plate was incubated for 15 min at 95°C , followed by 40 cycles of 15 s at 95°C and 1 min at 60°C . The gene dosage was deducted by calculating the difference in Ct from the Ct of the reference gene HPRT. The average Ct values for target genes were subtracted from the average housekeeping gene Ct values to yield the delta Ct. Results were expressed as $2^{-\Delta\text{Ct}}$ which is an index of the relative amount of gene expression.

Immunohistochemistry

Deparaffinised sections were stained with periodic acid–Schiff (PAS) to evaluate renal morphology. For immunostaining, sections were subjected to heat-induced antigen retrieval by overnight incubation in 0.1 M Tris/HCl buffer (pH 9.0) at 80°C . Endogenous peroxidase was blocked with 0.3% H_2O_2 in phosphate buffered saline (PBS, pH 7.4) for 30 min. Macrophages (ED1) and the pre-fibrotic marker for myofibroblast transformation alpha-smooth muscle actin (α -SMA) were detected using murine monoclonal antibodies (ED1; Serotec Ltd, Oxford, UK) (α -SMA; clone 1A4; Sigma). Binding was detected using sequential incubations with peroxidase-labelled rabbit anti-mouse (RAM^{po}) and peroxidase-labelled goat anti-rabbit (GAR^{po}) antibodies (Dakopatts, Glostrup, Denmark) for 30 min. All antibody dilutions were made in PBS supplemented with 1% BSA, and 1% normal rat serum was added to the secondary antibodies. Peroxidase activity was developed by using 3,3'-diaminobenzidine tetrachloride (DAB) for 10 min containing 0.03% H_2O_2 . Counterstaining was performed using Mayer's haematoxylin.

Analyses of histopathological changes

Focal glomerular sclerosis (FGS), interstitial and glomerular α -SMA expression and interstitial and glomerular macrophages were measured as described previously.¹²

Statistical analysis

Results are reported as mean \pm standard error of the mean. Statistical analysis among groups was performed with *T*-test if distributed normally or with Mann–Whitney *U* test

when skewed using non-parametric ANOVA (Kruskal–Wallis). For correction of multiple comparisons a Dunn's post-hoc analysis was performed. All *p*-values are two-tailed and a *p*-value of less than 0.05 was considered significant. All analyses were performed using SPSS version 18.0 software (SPSS, Chicago, IL, USA).

Results

Physiological parameters

All clinical research parameters are presented in Table 1. Body weight was in the same range for Ren2 and SD rats. DA and ACEi, nor the combination had any effect on body weight. Ren2 rats had a higher systolic blood pressure (SBP) during the entire study compared with SD rats (data not shown). DA had no effect on systolic blood pressure in both Ren2 and SD rats. ACE inhibition resulted in a reduced SBP, both in Ren2 and in SD rats. Ren2 rats showed lower haematocrit (ht) values when compared with SD rats at sacrifice (*p* < 0.05). DA treatment significantly increased ht in SD rats (*p* < 0.01), but only marginally in Ren2 rats (NS). There were no effects of ACE inhibition on ht. There was no significant difference in hemoglobin (Hb) in Ren2-VEH rats compared with SD-VEH rats, although SD rats showed a tendency towards higher Hb levels. DA treatment significantly increased Hb in SD rats (*p* < 0.05), but not in Ren2 rats. ACEi treatment significantly decreased Hb in Ren2 rats (*p* < 0.05). Serum creatinine was in the same range for Ren2 and SD rats. There were no effects of EPO DA or ACE inhibition on serum creatinine. Proteinuria was significantly increased in Ren2 versus SD (*p* < 0.01) DA treatment had no effect on proteinuria, while ACEi treatment significantly reduced proteinuria in Ren2 and Ren2-DA (*p* < 0.01).

Quantitative real-time PCR

Quantitative real-time PCR was performed for EPO, EPO-receptor, collagen-3α1 and KIM-1 relative to the housekeeping gene HPRT. DA treatment reduced EPO mRNA expression in both Ren2 rats (*p* < 0.05) and SD rats (*p* < 0.001) (Figure 1). EPO-receptor mRNA was significantly higher in Ren2 in comparison with SD (*p* < 0.001) (Figure 2). There were no effects of DA or ACEi treatment on EPO-receptor mRNA expression. Collagen-3α1 mRNA expression is significantly increased in Ren2-VEH compared with SD-VEH (*p* < 0.01). ACE inhibition in Ren2 resulted in a significant decrease of collagen-3α1 mRNA expression (*p* < 0.05) (Figure 3). KIM-1 expression was significantly increased in Ren2-VEH when compared with SD-VEH (*p* < 0.01). The combination of DA and ACEi significantly decreased KIM-1 expression in Ren2 rats versus Ren2-VEH (*p* < 0.05), while ACEi treatment alone did not (Figure 4).

Table 1. Clinical parameters at sacrifice

Parameter	Ren2-VEH, n = 13	Ren2-ACEi, n = 5	Ren2-DA, n = 14	Ren2-DA + ACEi, n = 5	SD-VEH, n = 12	SD-ACEi, n = 4	SD-DA, n = 12	SD-DA + ACEi, n = 4
Body weight (g)	308 ± 48.7	372 ± 15.7	323 ± 29.7	346 ± 26.6	378 ± 11.7	364 ± 27.3	347 ± 14.7	349 ± 15.2
Systolic blood pressure (mmHg)	216 ± 7.49	119.6 ± 6.96**	204.9 ± 1.1	122.5 ± 6.40**	139.6 ± 10.8*	118.6 ± 7.06#	143.0 ± 3.12	134.6 ± 10.5
Haematocrit (l/l)	0.37 ± 0.03	0.35 ± 0.03	0.43 ± 0.02	0.39 ± 0.02	0.44 ± 0.04*	0.43 ± 0.01	0.60 ± 0.04##	0.62 ± 0.00##
Haemoglobin (mmol/l)	9.05 ± 0.10	8.00 ± 0.16*	9.34 ± 0.14	8.40 ± 0.10	9.63 ± 0.77	10.03 ± 0.22	12.41 ± 0.18###	12.20 ± 0.21
Creatinine (µmol/l)	43.7 ± 7.1	35.8 ± 3.7	22.0 ± 14.4	32.6 ± 6.7	28.0 ± 3.1	27.3 ± 0.60	26.8 ± 1.7	20.8 ± 1.5
Proteinuria (mg/24 h)	84.6 ± 25.6	27.4 ± 4.1**	73.4 ± 11.5	28.9 ± 4.5**	15.3 ± 3.1**	22.1 ± 3.1	23.8 ± 6.9	15.9 ± 6.4

Data are presented as mean ± SEM.

p* < 0.05 vs. Ren-VEH, *p* < 0.01 vs. Ren2-VEH.

#*p* < 0.05 vs. SD-VEH, ##*p* < 0.01 vs. SD-VEH, ###*p* < 0.001 vs. SD-VEH.

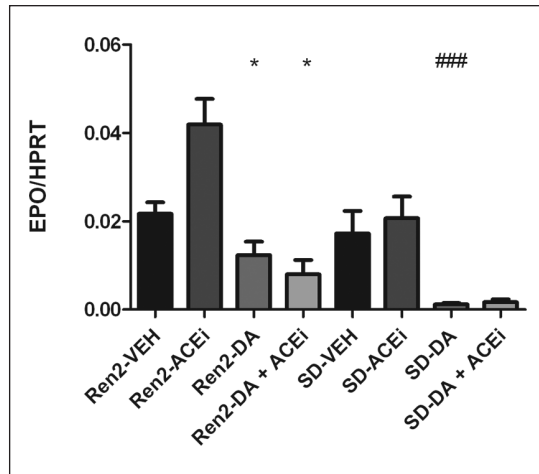


Figure 1. EPO mRNA expression in all groups at sacrifice. Data are presented as mean \pm SEM. *Ren2-VEH vs. Ren2-DA ($p < 0.05$), *Ren2-VEH vs. Ren2-DA + ACEi ($p < 0.05$), ###SD-VEH vs. SD-DA ($p < 0.001$).

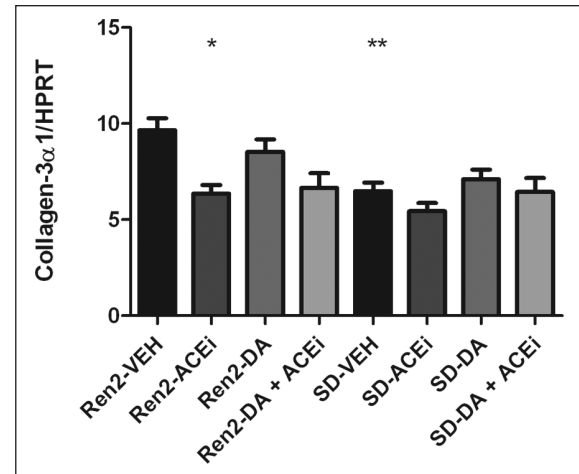


Figure 3. Collagen-3 α 1 mRNA expression in all groups at sacrifice. Data are presented as mean \pm SEM. *Ren2-VEH vs. Ren2-ACEi ($p < 0.05$), **Ren2-VEH vs. SD-VEH ($p < 0.01$).

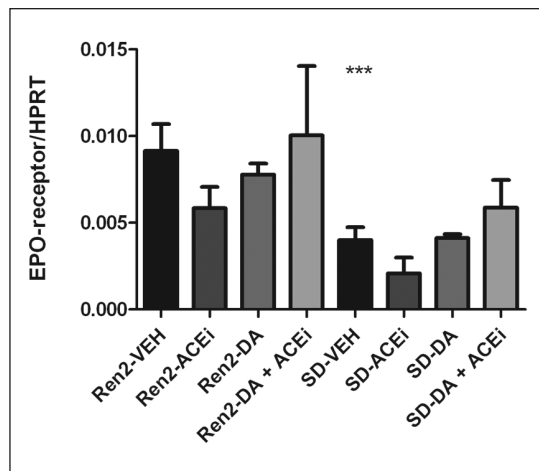


Figure 2. EPO-receptor mRNA expression in all groups at sacrifice. Data are presented as mean \pm SEM. ***Ren2-VEH vs. SD-VEH ($p < 0.001$).

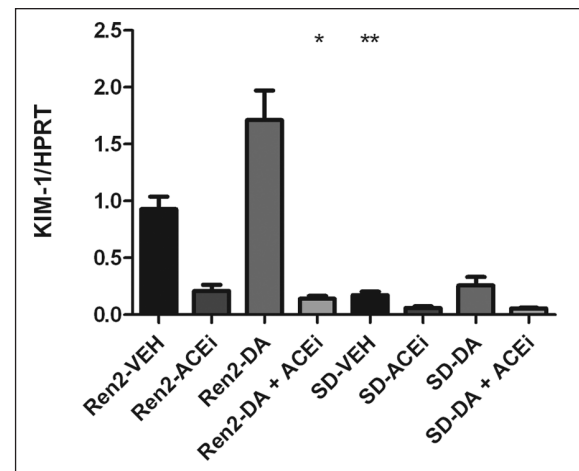


Figure 4. KIM-1 mRNA expression in all groups at sacrifice. Data are presented as mean \pm SEM. *Ren2-VEH vs. Ren2-DA + ACEi ($p < 0.05$), **Ren2-VEH vs. SD-VEH ($p < 0.01$).

Renal structural parameters

FGS was evident in Ren2-VEH and Ren2-DA rats. FGS was significantly higher in Ren2-VEH when compared with SD-VEH ($p < 0.001$). ACEi treatment, but not DA treatment, resulted in a significant decrease of FGS in all Ren2 rats ($p < 0.001$) (Figure 5a and b). There was a significant increase in renal interstitial damage in Ren2-VEH rats as evidenced by interstitial alpha smooth muscle actin (α -SMA) expression ($p < 0.0001$) (Figure 6). ACEi decreased the interstitial α -SMA expression in Ren2 ($p < 0.01$) and Ren2-DA ($p < 0.01$) when compared with Ren2-VEH, without ACEi treatment. Interstitial α -SMA expression was virtually absent in all SD groups. Glomerular α -SMA expression was significantly increased in Ren2 rats in comparison with SD rats ($p < 0.001$) and decreased in Ren2-DA + ACEi when compared with Ren2-VEH ($p < 0.05$). ACEi treatment alone did not decrease

glomerular α -SMA expression in Ren2 rats. Glomerular α -SMA expression was low in all SD groups (Figure 7). Interstitial macrophages were significantly increased in Ren2-VEH compared with SD-VEH ($p < 0.01$), while glomerular macrophages were significantly reduced in Ren2 rats ($p < 0.01$). ACEi treatment significantly reduced interstitial, but not glomerular, macrophages in Ren2 rats ($p < 0.001$). On neither of these parameters, DA treatment had any effects (Figures 8 and 9).

Discussion

The major finding of the present study is that long-term DA treatment has no beneficial effects on renal structural and functional changes induced by hypertension in TGR(mRen2)27 rats in the time frame studied and the dose provided.

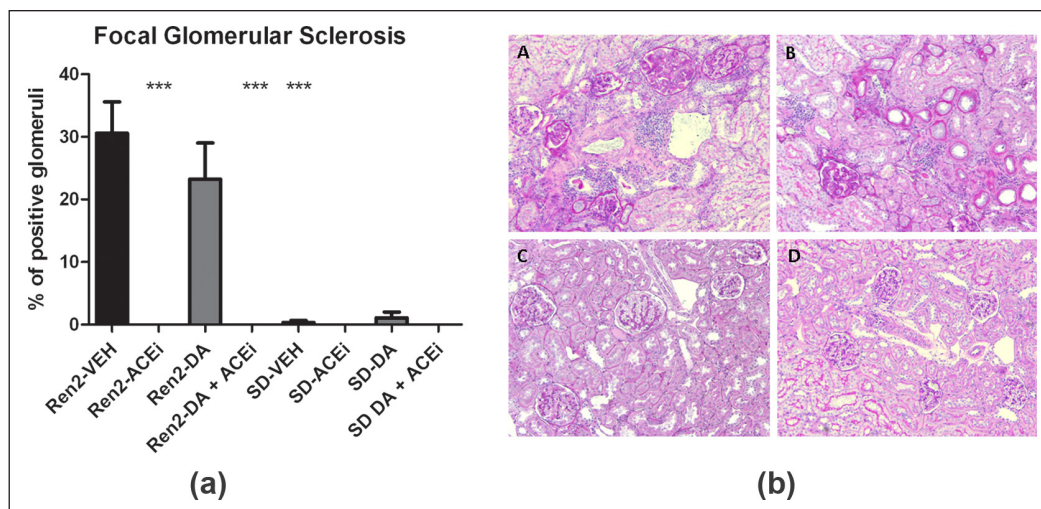


Figure 5. (a) FGS in all groups at sacrifice. Data are presented as mean \pm SEM. ***Ren2-VEH vs. Ren2-ACEi ($p < 0.001$), ***Ren2-VEH vs. Ren2-DA + ACEi ($p < 0.001$), ***Ren2-VEH vs. SD-VEH ($p < 0.001$). (b) Typical examples of PAS stained sections showing glomerular and interstitial damage in Ren2-rats and Ren2-DA. No damage was observable in SD controls. A. Ren2-VEH, B. Ren2-DA, C. SD-VEH, D. Ren2-DA + ACEi.

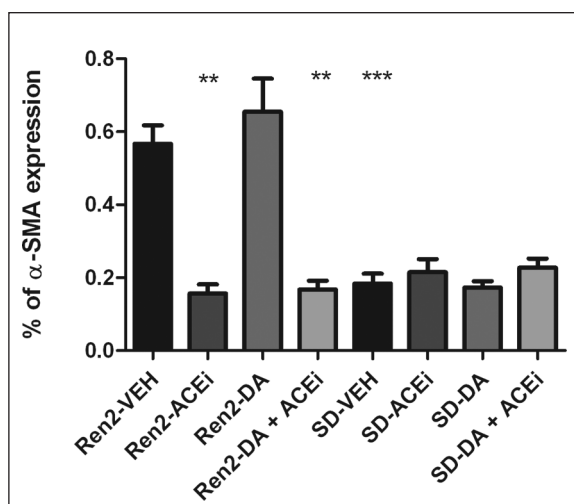


Figure 6. Interstitial α -SMA morphometry in all groups at sacrifice. Data are presented as mean \pm SEM. **Ren2-VEH vs. Ren2-ACEi ($p < 0.01$), **Ren2-VEH vs. Ren2-DA + ACEi ($p < 0.01$), ***Ren2-VEH vs. SD-VEH ($p < 0.001$).

Administration of DA in this model was effective as evidenced by a markedly reduced rat renal EPO mRNA expression both in Ren2 and SD. Despite similar treatment regiment in Ren2 and SD rats we noticed higher EPO-receptor mRNA expression in Ren2 rats suggesting a higher demand for DA reactivity during ischemic damage.

De Borst et al. previously described that the renal interstitial and glomerular changes in Ren2 rats are relatively mild.^{13,14} Surprisingly, glomerular macrophage influx was virtually absent in Ren2 rats. This lack of glomerular inflammation may be explained by a recent finding of our group showing a severe decrease in the number of burst

forming units of the erythroid lineage (BFU-E) in the bone marrow of Ren2 rats compared to SD, which suggests a suppressing role for either renin or angiotensin II in the activation of blood cell lineages.¹⁵

There is a discrepancy in haematocrit values between SD and Ren2 DA treated rats. We previously found that DA does not correct the ongoing anaemia in Ren2 rats as evidenced by stable haematocrit values. This suggests that there must be another player which accounts for the anaemia in Ren2 rats.¹⁵ We previously showed that Ren2 rats suffer from severe hypertension and as a consequence develop heart failure. DA treatment did not lead to better heart function or a decrease in damage in Ren2 rats.¹⁵

The cytoprotective properties of EPO in tissue are related to its binding capacity to a heterodimeric receptor complex, the EPOR2- β cR2 receptor. Binding to this receptor does not affect erythropoiesis. The tissue protective effects of EPO have been demonstrated pre- and post-perfusion in ischaemia/reperfusion models¹⁶⁻¹⁸. Numerous other studies in acute renal injury have revealed that EPO infusion preserves tissue and whole-organ function.^{9,19,20} However, only few studies have found similar protective effects of EPO treatment in the chronic setting. In 5/6 nephrectomy, chronic treatment with a haematologically non-effective dose of DA conferred renal vascular and tissue protection and preserved renal function.²¹ The effect was associated by reduced apoptotic cell death. However, escalating doses of DA mitigates the protective effects on the remnant kidney tissue and even worsens microvascular renal injury,^{21,22} suggesting that the dose is critical. In our study, DA had no evident effects on haematocrit and haemoglobin in treated Ren2 rats, and we therefore assume that the dose used was at least haematologically non-effective, as Bahlmann et al. describe in their study.²¹ Another

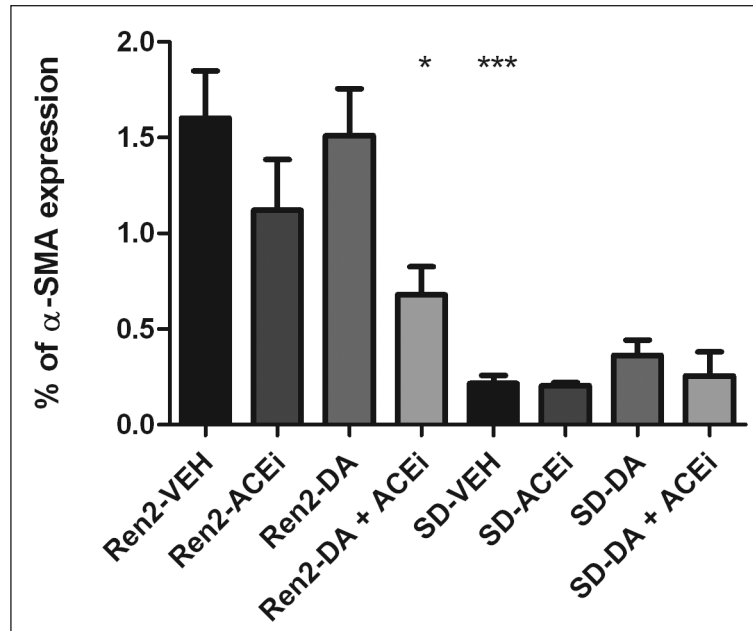


Figure 7. Glomerular α -SMA expression at sacrifice. Data are presented as mean \pm SEM. *Ren2-VEH vs. Ren2-DA + ACEi ($p < 0.05$), ***Ren2-VEH vs. SD-VEH ($p < 0.001$).

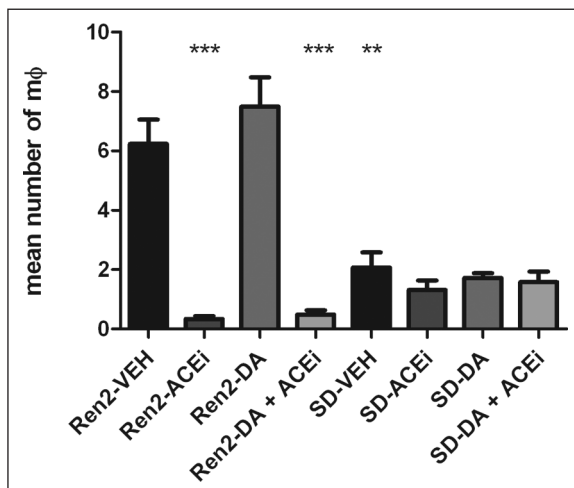


Figure 8. Number of interstitial macrophages in all groups. Data are presented as mean \pm SEM. ***Ren2-VEH vs. Ren2-ACEi ($p < 0.001$), ***Ren2-VEH vs. Ren2-DA + ACEi ($p < 0.001$), **Ren2-VEH vs. SD-VEH ($p < 0.01$).

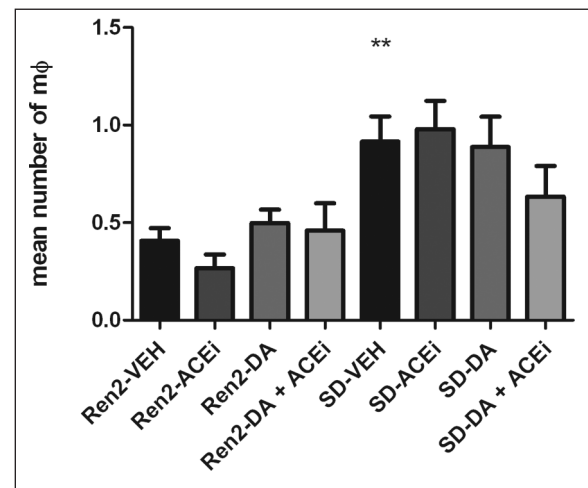


Figure 9. Number of glomerular macrophages in all groups. Data are presented as mean \pm SEM. **Ren2-VEH vs. SD-VEH ($p < 0.01$).

difference is that in our study rats were treated once in 3 weeks with DA, while Bahlmann et al. administered DA weekly.²¹ The treatment regiment used in this study was based on previous positive experience with DA treatment in the experimental setting in SD rats, where this dose of DA effectively raised haematocrit.^{23,24} It may well be that one dose of DA every 3 weeks is less effective than when DA is administered more frequently and plasma concentrations of

DA are being kept at a more constant level during the entire experiment.

A possible limitation of our study is that it is not sure if the renal damage induced by the transgenic phenotype in Ren2 rats was severe enough for DA to exert its beneficial effects. DA may be more effective in conditions with extensive damage and thus more ischaemia. In our experimental model the renal structural and functional changes

were relatively mild. This is probably due to the compulsory sacrifice of the rats after 6 weeks of treatment. If the rats were not sacrificed at this time point, they would have died spontaneously at 14 weeks of age due to vascular complications.

Conclusion

From this study, we conclude that long-term DA treatment has no beneficial effects on renal structural and functional changes induced by hypertension in Ren2 rats in the time frame studied and the dose provided. However, this does not exclude a role for this growth factor in chronic renal disease, since the observed changes in our hypertensive model were relatively mild. Therefore, further studies are needed to elucidate a possible role for DA in renal disease.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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