Original Articles

Long-term mineralocorticoid receptor blockade ameliorates progression of experimental diabetic renal disease

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Abstract
The final end point of diabetic renal disease is the accumulation of excess collagen. A number of studies have shown that aldosterone antagonism ameliorates progression of renal fibrosis. This study was designed to examine the effect of the mineralocorticoid receptor blocker eplerenone (EPL) on progression in streptozotocin (STZ)-treated spontaneously hypertensive rats (SHR), an accelerated model of Type I diabetes. STZ-treated SHRs with a blood glucose >18 mmol/L were randomly divided into treatment (100 mg/kg/day EPL) and non-treatment groups. Sham-injected SHR animals were used as a control. Functional parameters were monitored for 16 weeks, with structural parameters assessed at completion. Both hyperglycaemic groups developed progressive albuminuria, but the increase was ameliorated by EPL from Week 12. STZ–SHRs had elevated kidney weight/body weight ratio, glomerular size, glomerular macrophages (ED-1-positive cells), tissue transforming growth factor beta 1 (TGFβ1) concentrations and glomerular collagen IV staining (all P < 0.05 versus control animals). EPL reduced glomerular volume, TGFβ1 expression and glomerular collagen IV without changing glomerular macrophage infiltration. The ability of EPL to ameliorate these functional and structural changes in hyperglycaemic SHRs suggest that EPL has a renoprotective role in diabetic renal disease.

Keywords: albuminuria; aldosterone; diabetic nephropathy; fibrosis

Introduction
Diabetic nephropathy is the leading cause of chronic renal failure leading to end-stage renal disease in many countries worldwide [1]. The formulation of strategies to prevent the development of diabetic nephropathy is therefore an important goal of renal research.

The onset of diabetic nephropathy is heralded by the development of albuminuria, with histological changes characterized by expansion of the glomerular mesangial matrix. Multiple animal and clinical studies have shown that the use of inhibitors of the renin–angiotensin–aldosterone system (RAAS) is associated with a decrease in progression of diabetic nephropathy indicating that the RAAS plays a central role in diabetic renal disease [2]. However, the widespread use of angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs) in hypertensive and proteinuric renal disease has resulted in the observation that despite continuing these drugs, an initial fall in plasma aldosterone levels is only transient in a high proportion of patients—a phenomenon known as aldosterone breakthrough [2]. Although the mechanism for aldosterone breakthrough is currently obscure, as is the source of the aldosterone, a growing body of evidence has highlighted the pathogenic role of aldosterone in renal fibrosis [3].

Again, recent clinical [4–8] and experimental studies [9] suggest that aldosterone antagonism, through mineralocorticoid receptor blockade, ameliorates progression of diabetic renal complications. The mechanism is obscure but based on non-diabetic studies is thought to include both direct and indirect cellular effects and reductions in proteinuria. Progress in elucidating these mechanisms has been slow because the animals models used thus far have only approximated the pathophysiology of diabetic nephropathy.

In this study, we have examined the therapeutic potential of aldosterone blockade in diabetic renal complications by measuring the effect of eplerenone (EPL) on progression in a hypertensive rat model of streptozotocin (STZ)-induced diabetes. EPL is a relatively new selective aldosterone antagonist, thought to have a lower incidence of adverse effects when compared to the older drug spironolactone [10]. The hypothesis tested was that EPL ameliorates the pathophysiology of diabetic renal disease. The STZ-treated spontaneously hypertensive rat (SHR) was chosen as it is an accelerated model that rat mimics many of the changes seen in human diabetic complications [11]. In addition, the SHR has been shown to exhibit aldosterone breakthrough with angiotensin receptor blockade [12], making it an ideal experimental background for these studies.
Materials and methods

Animals

Six-week-old female SHRs were randomized to receive either 55 mg/kg STZ (Sigma, St Louis, MO) diluted in 0.1 M citrate buffer (pH 4.5) or citrate buffer alone (for non-diabetic controls), by tail-vein injection after an overnight fast. In each subsequent week, rats were weighed and blood glucose levels were determined (AMES glucometer; Bayer Diagnostics, Melbourne, Australia). Diabetic rats were defined as those with a blood glucose >18 mmol/L, 2 weeks after STZ injection. Diabetic animals were further randomly allocated to treatment control (untreated) and treatment groups (n = 6–7 per group). Treatment animals had drinking water supplemented with EPL, with the dose titrated to deliver –100 mg/kg/day over the course of the experiment. All rats were housed in a controlled environment (maintained at 22 ± 1°C with a 12-h light/dark cycle) and had free access to rodent lab chow and water throughout the experiment. Diabetic animals received insulin (Human Insophane; Eli Lilly, West Ryde, Australia; 2 U/day) as required to maintain body weight and avoid ketonuria without achieving euglycaemia. These experiments were approved by the Royal Children’s Hospital Animal Ethics Committee, which adheres to the Australian and New Zealand College of Toxicology, Birmingham, AL), goat-anti-collagen I (Biodesign International, Saco, ME), mouse anti-rat ED1 (Serotec, Kidlington, UK), mouse anti-proliferating cell nuclear antigen (PCNA; Dako) followed by species matched biotinylated anti-IgG, and deflation of the cuff, where SBP was defined as the inflation pressure at which the waveform became indistinguishable from baseline noise. Final SBP readings were obtained by averaging three successful readings.

Renal functional measurements and tissue collection

At Weeks 0, 1, 4 and every 4 weeks thereafter, a 24-h urine collection was made from conscious diabetic and non-diabetic rats for measurement of albuminuria by ELISA assay (Bethyl Laboratories, Montgomery, TX). Rats were then anaesthetized lightly with an inhalational anaesthetic (Metohylxurane; Abbott Laboratories, Sydney, Australia), and blood collected via tail vein for measurement of blood glucose at the same time points, glycated haemoglobin A1c (HbA1c) levels at Week 16 and serum creatinine at Weeks 0, 8 and 16 (both measured by automated methods). After 16 weeks, animals were killed by an overdose of anaesthetic before the left kidney was weighed and sliced transversely for histochemistry.

Serum aldosterone concentration

Serum samples collected from animals in each group at Weeks 0 and 16 were assayed for circulating aldosterone, using a commercially available Aldosterone ELISA kit (Alpha Diagnostic International Inc, San Antonio, TX). Briefly, samples and standards along with avidin conjugate solution were added to wells and left to incubate at room temperature for 60 min. Wells were then washed and horseradish peroxidase substrate was added for a further 15 min before stop solution was added to the wells, and optical density was read at 450 nm. Each sample was assayed in duplicate. This assay has previously been shown to detect rat aldosterone [13], is specific and sensitive, with the minimal detectable concentration estimated to be 15 pg/mL.

Tissue collection and histochemistry

Fixed tissue was routinely processed, embedded in paraffin and sectioned. Sections were labelled with mouse goat-anti-collagen IV (Southern Biotechnology, Birmingham, AL), goat-anti-collagen I (Biodesign International, Saco, ME), mouse anti-rat ED1 (Serotec, Kidlington, UK), mouse anti-desmin (Dako, Glostrup, Denmark) or mouse anti-proliferating cell nuclear antigen (PCNA; Dako) followed by species matched biotinylated anti-IgG, as appropriate. Binding was visualized with avidin–biotin complex (ABC reagent (according to the manufacturer’s instructions; Life Technologies, Gaithersburg, MD), analysed with a polyclonal antibody to transforming growth factor beta 1 (TGFβ1) (sc-146, Santa Cruz Biotechnology, Santa Cruz, CA) and detected using an appropriate secondary antibody (Cell Signaling Technology, Danvers, MA). Densitometry of TGFβ1 dimer (25 kDa) bands was performed using a Bio-Rad GS710 Calibrated Imaging Densitometer and Quantity-One™ software (Bio-Rad, Richmond, CA). The density of TGFβ1 was expressed relative to coomassie blue-stained total protein. As sodium dodecyl sulphate-polyacrylamide gel electrophoresis has been shown to activate latent TGFβ1 [17], these western blots approximate total TGFβ1 levels.

Statistical analysis

Discrete data were analysed by a one-way analysis of variance (ANOVA), using the Bonferroni post-hoc test for multiple comparisons between groups. Inter-group and Intra-group comparisons were made using two-way ANOVA, with post-hoc analysis used to isolate longitudinal and treatment differences. Serum albumin concentrations at Weeks 0 and 16 were compared by paired t-test. Data in this paper are presented as the mean ± standard error of the mean, with P < 0.05 considered statistically significant.

Results

Characteristics of the experimental model

STZ-treated SHRs with blood glucose >18 mmol/L at Week 1 were randomly allocated to treatment (SHR–STZ EPL) and non-treatment (SHR–STZ) groups. In each case, animals had sustained hyperglycaemia >16 weeks, with no difference between experimental groups (Figure 2A). Conversely, animals in the sham-injected (control) group were normoglycaemic (Figure 2A). At sacrifice, plasma glucose

B

SHR

SHR-STZ

SHR-STZ EPL

Fig. 1. Extracellular matrix changes in the experimental model. (A) Semi-quantitative grading of glomerular collagen IV based on immunostaining. (B) Conversely, in each group, collagen I staining was confined to the interstitial space (arrows) and not seen in glomeruli. Scale bar = 50 μm.
levels and HbA1c glycation were ~5-fold and 2-fold higher in diabetic animals than their non-diabetic counterparts, respectively (Figure 2A and B; both $P < 0.001$). Long acting insulin was used to maintain body weight and well-being in the diabetic animals. Consistent with this, body weight increased progressively in the sham (control) group, with no change in the two groups of diabetic animals (Figure 2C).

Circulating aldosterone concentrations (pg/mL) increased over time ($P < 0.05$; Week 16 versus Week 0) in each of the experimental and control groups (Figure 3).

**Functional changes**

Untreated diabetic animals developed proteinuria, as indicated by a progressive rise in albumin excretion (Figure 4A). Albuminuria in the treated animals paralleled that seen in the EPL-treated group for the first 8 weeks, after which there was a significant divergence (Figure 4A; $P < 0.05$ SHR–STZ EPL versus SHR–STZ at Weeks 12 and 16). There was an acute
increase in serum creatinine concentration post-STZ injection (P < 0.05 Week 1 SHR–STZ EPL and SHR–STZ versus SHR), but this difference between groups was not sustained, with no difference between groups at the experimental endpoint (Figure 4B).

Experimental groups were well matched for starting blood pressure. Animals all remained hypertensive throughout, with no difference between groups (Figure 4C).

**Effects of hyperglycaemia and EPL treatment on organ hypertrophy**

Weight of the left kidney and glomerular volume were used as measures of renal hypertrophy (Figure 5). Kidney weight, corrected for body weight, was greater in the SHR–STZ animals than the sham control group (P < 0.05; Figure 5A). This difference was not seen in the EPL group (SHR–STZ EPL versus SHR). Although glomeruli volume in diabetic animals were not larger than their sham controls, EPL treatment was associated with a reduction in glomerular volume (*P < 0.05 versus SHR–STZ, P = n.s. versus SHR), suggesting a trend towards less glomerular hypertrophy.

**Effects of hyperglycaemia and EPL treatment on organ pathology**

Accumulation of glomerular collagen remains the histological hallmark of progression in diabetic renal disease (Figure 1A). At the completion of the experiment (Week 16), diabetic SHRs had similar levels of total collagen IV to sham (control) animals (% fractional area) (Figure 6A) but increased glomerular collagen IV staining index (Figure 6B; P < 0.05 versus control animals). Collagen I staining was confined to the interstitium and not seen in the glomerulus (Figure 1B). Sixteen weeks of EPL treatment had no marked effect on total collagen IV but reduced glomerular collagen IV staining to the levels seen in sham controls (Figure 6B; P < 0.05 versus SHR–STZ, P = not significant versus SHR).

Immunohistochemical staining for ED-1 was used as a marker of renal macrophages and hence inflammation. The number of ED-1-positive cells per glomerular profile was estimated morphometrically. Glomerular macrophages were seen in all groups, consistent with the pathophysiology of underlying spontaneous hypertension and diabetes. The SHR–STZ and SHR–STZ EPL groups had on average 5.1 ± 0.4 and 4.5 ± 0.2 macrophages per glomerulus, respectively, significantly more than the sham animals (3.3 ± 0.2; both P < 0.05), with no difference between the treated and untreated groups (Figure 7).

Glomerular staining for desmin was likewise used as a surrogate marker of podocyte injury [18] (Figure 8). Glomerular desmin staining was equivalent in sham (2.95 ± 1.20), control (2.73 ± 1.16) and treatment groups (2.83 ± 1.15). Glomerular proliferation was assessed by enumerating cell expression of proliferating cell nuclear antigen (PCNA). The incidence of PCNA staining was low, with on average only one PCNA-positive cell in each glomerulus, in all three groups (1.1 ± 0.2, 1.0 ± 0.1, 1.1 ± 0.2 in sham, control and treatment groups, respectively) (Figure 9). There was therefore no quantitative difference between groups.

![Fig. 5. Measures of renal hypertrophy](image)

![Fig. 6. Collagen IV accumulation](image)
Western blotting for TGFβ1 was used to measure local concentrations of this key pro-fibrotic cytokine. STZ treatment increased total TGFβ1 concentrations (active and latent peptide) 4-fold (P < 0.001; SHR–STZ versus SHR). EPL treatment resulted in a significant reduction in TGFβ1 expression (P < 0.001; SHR–STZ EPL versus SHR–STZ), although tissue levels were still higher than that seen in non-diabetic controls (P < 0.05; SHR–STZ EPL versus SHR–STZ) (Figure 10).

Discussion

Although the pathophysiology is complex, it is well recognized that renal complications progress in diabetes. This study specifically examined the renoprotective properties of a specific mineralocorticoid receptor (MR) blockade in an experimental model of diabetic renal complications. Our principal findings were that long-term administration of EPL ameliorated the rise in proteinuria (albuminuria) and glomerular collagen IV, which was paralleled by a decrease in TGFβ1 expression.

Diabetic nephropathy develops in ~40% of patients with diabetes [19] and is the leading single cause of end-stage renal failure [20]. However, diabetic nephropathy is a human condition, with animal models only approximating its pathophysiology. The natural history of our experimental model was consistent with early diabetic renal disease, sustained hyperglycaemia, hypertension and proteinuria with modest renal hypertrophy and glomerulopathy, in the absence of any tubulointerstitial pathology. Serum creatinine did not change, but in isolation, it is a relatively insensitive measure of renal function.

Despite the significance of progressive sclerosis and fibrosis, therapeutic strategies for its treatment remain elusive. Although ACEi, ARBs and beta-blockers are now...
after 8 weeks, but not before, was unexpected. The delay why MR blockade should ameliorate the rise in proteinuria the final 8 weeks of the experiment. Histological analysis by treatment with EPL, although this effect was confined to increased collagen IV staining. Albuminuria was ameliorated Glomeruli in diabetic animals were hypertrophied, with in- progressive rise in albuminuria over the 16-week period. Not developed proteinuria. Because of different experimental groups have used MR blockade in animal models of Type I 3–4 weeks after STZ administration [9, 26], when animals had largely focused on acute changes in normotensive models [9], these studies have provided valuable insights, they have Viewed as first line treatment for diabetic nephropathy and cardiomyopathy [21] and have clearly been shown to confer organ protection, diabetes remains a progressive disorder, which ultimately leads to organ failure. Indeed, captopril, although retarding the decline in renal failure, did not halt the progression of diabetic nephropathy in the vast majority of patients [22]. It is therefore imperative that we identify novel interventions. Several recent studies have examined the efficacy of the aldosterone antagonists spironolactone and EPL in experimental renal disease. Spirolactone has ameliorated progression of fibrosis in a diverse range of primary renal diseases including thy-1 nephritis [23], adriamycin nephritis [24] and acute cyclosporin A nephrotoxicity [25]. Likewise, several groups have used MR blockade in animal models of Type I [9, 26] and II [27, 28] diabetic complications. These have shown that MR blockade reduces fibrogenesis (collagen IV messenger RNA expression) [28] and early fibrosis [9]. While these studies have provided valuable insights, they have largely focused on acute changes in normotensive models [9], 3–4 weeks after STZ administration [9, 26], when animals had not developed proteinuria. Because of different experimental objectives, proteinuria and glomerular pathology have often not been analysed [26]. In this context, our findings are both in agreement and an extension of recent work. The natural history of this model was associated with a progressive rise in albuminuria over the 16-week period. Glomeruli in diabetic animals were hypertrophied, with increased collagen IV staining. Albuminuria was ameliorated by treatment with EPL, although this effect was confined to the final 8 weeks of the experiment. Histological analysis showed that this was accompanied by a reduction in collagen IV and a trend towards less glomerular hypertrophy. Why MR blockade should ameliorate the rise in proteinuria after 8 weeks, but not before, was unexpected. The delay does, however, seem to suggest multiple mechanisms in the pathogenesis of diabetic complications. The amelioration of proteinuria after 8 weeks is consistent with progression being due initially to diabetic-specific factors, with a more prominent role for aldosterone later on. Consistent with this, each experimental arm of our study showed a rise in aldosterone levels over time (Weeks 0–16). This may well explain why EPL had a greater effect in the later stages of this study (Weeks 12–16). Future studies are needed to understand the role of aldosterone levels, local aldosterone production and receptor expression in this. Indeed, Nelson and Tuttle [29] have recently highlighted that a growing number of clinical trials refute the widely held belief that RAAS blockade is of benefit in all stages of diabetic kidney disease. Controversy surrounds the mechanism by which aldosterone antagonists ameliorate progression in general and proteinuria in particular. The effect is not generally proportionate to observed reductions in blood pressure [2]. In the current study, animals were well matched for blood pressure at the start of the experiment, and 16 weeks of EPL treatment had no anti-hypertensive effect. This is consistent with cardiac studies in ageing SHRs [30], where spironolactone had very little effect on arterial pressure. Although we did not perform more detailed studies of intra-glomerular haemodynamics, the vasoconstrictor actions of aldosterone are thought to be unaffected by spironolactone [31]. The presence of MR on podocytes [25], mesangial cells [32] and fibroblasts [33] suggests direct anti-fibrotic effects. The reduction in glomerular collagen IV staining is consistent with this, collagen IV being an indirect marker of mesangial cells. An aldosterone-mediated podocyte injury has been demonstrated, independent of blood pressure [25]. Although a similar detailed analysis of podocyte ultrastructure was beyond this study, we did not see any change in expression of glomerular desmin, a surrogate marker of glomerular epithelial cell injury [18]. Several studies have also shown that aldosterone blockade may slow progression by ameliorating inflammation [34]. Although we saw a STZ-dependent increase in glomerular macrophages, this was not altered by EPL. Aldosterone infusion increases mesangial cell proliferation in vivo [35] via mitogen-activated protein kinase (MAPK) activation [36]. Hyperglycaemia in isolation did not increase proliferation above that seen in the naïve SHR group, commensurate with the slow pathogenesis of diabetic glomerulopathy and suggesting that the experiment was underpowered to detect changes in cell cycling at any single time-point. Nonetheless, once again, we were unable to demonstrate any change in glomerular proliferation with EPL. The measurement of TGFβ1 levels in these animals does, however, provide a mechanistic rationale. Exacer- bated TGFβ1 expression by hyperglycaemia is central to the pathogenesis of diabetic renal complications. The amelioration of this increase is in agreement with Yuan et al. [37] and suggests that EPL’s functional and structural effects are mediated by a reduction in TGFβ1 signalling. This study is clearly not without limitations, the time course is still relatively short compared to the human time frame, and this is clearly reflected in the modest pathological changes seen. Again all animal models, including the one here, are only an approximation of the human condition.
Furthermore, future studies would benefit from more detailed studies of podocyte pathology and more sophisticated measures of blood pressure and glomerular hemodynamics. The downstream effects on TGFβ1 signalling remain to be elucidated. However, notwithstanding this, we believe our study has important clinical implications. Aldosterone blockade is increasingly advocated clinically, even though its mechanism of action is poorly understood. Our study is a logical extension of recent work and has provided useful insights in a clinically relevant model of Type I diabetic complications, the hypertension STZ–SHR.

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