Effect of eplerenone, enalapril and their combination treatment on diabetic nephropathy in type II diabetic rats

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Abstract
Background. Recent data suggest that aldosterone antagonists have beneficial effects on diabetic nephropathy. In this study, we investigated the dose-dependent effect of eplerenone and a combined treatment with eplerenone and enalapril compared with each drug alone on renal function in type II diabetic rats. To further explore the molecular mechanism of action of combination therapy, we also performed in vitro study.

Methods. The animals were divided into six groups as follows: normal control Long-Evans Tokushima Otsuka (LETO) rats, Otsuka Long-Evans Tokushima Fatty (OLETF) rats, OLETF rats treated with low dose of eplerenone (50 mg/kg/day), OLETF rats treated with high dose of eplerenone (200 mg/kg/day), OLETF rats treated with enalapril (10 mg/kg/day) and OLETF rats treated with a combination of both drugs (eplerenone 200 mg/kg/day and enalapril 10 mg/kg/day) for 6 months.

Results. Treatment of OLETF rats had no significant effect on body weight, kidney weight and blood glucose levels. However, urinary albumin excretion, glomerular filtration rate and glomerulosclerosis were significantly improved in the enalapril group and improvement was observed in a dose-dependent manner in the eplerenone groups; the most dramatic decreases were observed in the combination group. In accordance with these findings, renal expressions of TGF-β₁, type IV collagen and PAI-1 were also markedly decreased in the treatment groups, with the combined treatment providing the most significant level of improvement. In cultured mesangial cells, combined treatment resulted in an additive decrease in TGF-β₁, PAI-1 and collagen gene expressions and protein production induced by high glucose and aldosterone stimulation.

Conclusions. Aldosterone receptor antagonism provided additional benefits beyond blockade of the renin–angiotensin system in type II diabetic nephropathy.

Keywords: albuminuria; diabetic nephropathy; enalapril; eplerenone; glomerulosclerosis

Introduction

The renin–angiotensin–aldosterone system (RAAS) has been a focus of research in cardiovascular, renal and atherosclerotic diseases for several decades [1]. Extensive research has found that the blockade of RAAS has a widespread protective role by producing anti-inflammatory, anti-proliferative and anti-oxidative effects [1–6]. Currently, angiotensin II is considered the primary mediator of RAAS, and thus agents that block RAAS, such as angiotensin-converting enzyme inhibitors (ACEI) or angiotensin II receptor antagonists (ARB), have been developed as powerful strategies to slow the progression of renal diseases [7]. However, despite the beneficial effects of these strategies, their effects are limited with respect to the prevention of end-stage renal failure.

Previous reports have demonstrated that the administration of spironolactone, a nonselective aldosterone blocker, has beneficial effects in a variety of animal models of renal injury such as the unilateral ureteral obstruction model, cyclosporin nephrotoxicity model and hypertensive renal injury model [8–10]. In addition, recent data have demonstrated improved renal function in streptozotocin-induced diabetic rats, with clinical trials suggesting the possibility that inhibition of the aldosterone system may have additional beneficial effects independent of renin–angiotensin blockade on diabetic nephropathy [11–14].

Recent evidence also indicates that aldosterone inhibition may have additional renoprotective effects in patients with early diabetic nephropathy who experience the aldosterone escape phenomenon during ACE inhibitor treatment [15]. Another recently published study has shown that in patients with chronic renal disease and persistent proteinuria of > 0.5 g/day, despite ideal BP control with an ACE inhibitor, adding spironolactone to the conventional treatment produces beneficial effects on urinary protein excretion,
especially in diabetic patients [16]. Therefore, inhibition of the aldosterone system as well as the renin–angiotensin system (RAS) raises the possibility that a dual blockade of RAS and aldosterone may result in a more complete blockade of RAAS than the inhibition of either system alone, providing individuals with increased beneficial effects on the prevention of progression of diabetic nephropathy. However, it is still unknown whether combined treatment with aldosterone receptor antagonist based on RAS inhibition provides increased renal protective effects on the diabetic nephropathy of a type II diabetic animal model.

For these reasons, we investigated whether different doses of eplerenone result in a different effect in renal protection, and tested that combined therapy of eplerenone and enalapril provides a more beneficial effect compared with each treatment alone on the renal function in type II diabetic nephropathy. In addition, we conducted an in vitro study in order to elucidate a renal protective mechanism of dual blockade, and we evaluated the effect of dual blockade on high glucose and aldosterone-induced synthesis of TGF-β1, type IV collagen and PAI-1 in cultured mesangial cells.

Materials and methods

Animals

Male OLETF rats, a model of type II diabetes mellitus, were kindly supplied by the Tokushima Research Institute (Otsuka Pharmaceutical Co, Tokyo, Japan). Male Long-Evans-Tokushima-Fatty (LETO) rats served as a genetic control. All rats were maintained at a controlled temperature (23 ± 2°C) and humidity (55 ± 5%) under an artificial light cycle. Animals were given free access to rat chow. At 20 weeks of age, rats were divided into six groups (n = 6/group). Group 1 consisted of LETO control rats, group 2 consisted of OLETF type II diabetes rats, group 3 consisted of OLETF rats treated with low dose of eplerenone at a dose of 50 mg/kg/day (Pfizer Inc., New York, USA) mixed in rat chow, group 4 consisted of OLETF rats treated with high dose of eplerenone at a dose of 200 mg/kg/day mixed in chow, the fifth group consisted of OLETF rats treated with 10 mg/kg/day of enalapril in drinking water (Sigma-Aldrich, St Louis, MO, USA) and the sixth group consisted of OLEFT rats treated with both 10 mg/kg/day of enalapril and 200 mg/kg/day of eplerenone. Administration of enalapril or eplerenone was begun at 20 weeks of age, rats were divided into six groups (n = 6/group). Group 1 consisted of LETO control rats, group 2 consisted of OLETF type II diabetes rats, group 3 consisted of OLETF rats treated with low dose of eplerenone at a dose of 50 mg/kg/day (Pfizer Inc., New York, USA) mixed in rat chow, group 4 consisted of OLETF rats treated with high dose of eplerenone at a dose of 200 mg/kg/day mixed in chow, the fifth group consisted of OLETF rats treated with 10 mg/kg/day of enalapril in drinking water (Sigma-Aldrich, St Louis, MO, USA) and the sixth group consisted of OLEFT rats treated with both 10 mg/kg/day of enalapril and 200 mg/kg/day of eplerenone. Administration of enalapril or eplerenone was begun at 20 weeks of age, which is the time usually required to develop overt hyperglycaemia in OLETF rats. The treatment regimen was maintained for 6 months. Daily amounts of food and water intake were checked at regular intervals to affirm the dose of administered drug. Plasma glucose levels were measured using a glucose oxidase-based method, plasma potassium was measured by flame photometry and serum creatinine levels were determined using a modified Jaffe method. The amount of urinary albumin was determined with a competitive ELISA kit (Shibayagi, Shibukawa, Japan). Urinary albumin concentrations were normalized to urinary creatinine concentrations. Creatinine clearance was used to estimate glomerular filtration rate, and was calculated on the basis of plasma and urinary creatinine concentrations and the corresponding urine volume; it is expressed as milliliters per minute per 100 grams body weight. At the end of the study period, systolic blood pressure was measured using tail-cuff plethysmography (LE 5001-Pressure Meter, Letica SA, Barcelona, Spain). Rats were killed under anaesthesia by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg body weight. Experiments were conducted in accordance with the Korea University Guide for Laboratory Animals.

Determination of serum and urinary concentrations of βig-h3

Serum and urine concentrations of transforming growth factor-β inducible gene-h3 (βig-h3) were measured with ELISA as previously described [17]. Briefly, 96-well flat-bottom plastic microtitre plates (Coster, Cambridge, MA, USA) were coated with 0.5 μg/mL of wild-type recombinant βig-h3 protein in a 20 mM carbonate–bicarbonate buffer (pH 9.6) with 0.02% sodium azide overnight at 4°C. The plates were then rinsed three times in PBS-0.05% Tween-20 (PBST) and maintained at 4°C. Lyophilized culture media was then pre-incubated with anti-βig-h3 antibodies (diluted 1:2000 in PBST) in 96-well round-bottom plastic microtitre plates for 90 min at 37°C. The pre-incubated samples were then transferred to the pre-coated plates and incubated for 30 min at room temperature. The plates were rinsed three times in PBST and incubated for 90 min at room temperature with the peroxidase-conjugated anti-rabbit IgG antibodies (diluted 1:2000 in PBST, Amersham, Arlington, IL, USA). Thereafter, the plates were rinsed again three times in PBST and incubated for 60 min at room temperature in the dark with a substrate solution (prepared by dissolving O-phenylenediamine in methanol at a concentration of 10 mg/mL, diluting 1:100 with deionized water and adding 0.01 mL of 30% H2O2 per 100 mL of the solution). After termination of the reaction with 8 N H2SO4, the absorbance was read at 492 nm. The detection limit of βig-h3 was 10 ng/mL. Concentrations of urinary βig-h3 were normalized to urinary creatinine concentrations.

RNA extraction and analysis of gene expression by real-time quantitative PCR in renal tissues and mesangial cells

Total RNA was extracted from renal cortical tissues or experimental cells with Trizol reagent and further purified using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instruction. The nucleotide sequence of each primer was as follows: TGF-β1, sense 5′-ATA CAG GGC TTT CGA TCC AGG-3′ and anti-sense 5′-GTC CAG GCT CCA AAT ATA GG-3′; type IV collagen, sense 5′- TAG GTG TCA GCA ATT AGG CAG G-3′ and anti-sense 5′-CGG ACC ACT ATG CTT GAA GTG A-3′; PAI-1, sense 5′-ATG AGA TCA GTA CTG CGG ACG CCA TCT TTG-3′ and anti-sense 5′-GCA CGG AGA TGG TGC TAC CAT CAG ACT TGT-3′; β-actin, sense 5′-TCA TGA GTG AGT CCG TCA GG -3′ and anti-sense 5′-TCT AGG CAC CAA GGT GTG -3′. Quantitative gene
expression was performed using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA, USA) using SYBR Green technology. Total mRNA was reverse-transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The specificity of each PCR product was evaluated by melting curve analysis, followed by agarose gel electrophoresis to confirm the presence of a single clean band. Real-time RT-PCR was performed by heating reactions for 10 min at 50°C and 5 min at 95°C, followed by 45 cycles consisting of denaturation for 10 s at 95°C and annealing with extension for 30 s at 60°C. At the end of the PCR cycle, samples were heated to 95°C to confirm the presence of a single, specific PCR product. The mRNA level of each sample was normalized to that of β-actin mRNA.

Histological examination

Paraffin-embedded kidney tissues were cut into 4-µm-thick slices and stained with periodic acid-Schiff (PAS). A semi-quantitative score (SI) was used to evaluate the degree of glomerulosclerosis on PAS-stained sections according to the method described by Ma et al. [18]. The severity of sclerosis for each glomerulus was graded from 0 to 4+ as follows: 0, no lesion; 1+, sclerosis of <25% of the glomerulus; 2+, 3+ and 4+, sclerosis of 25 to 50%, >50 to 75% and >75% of the glomerulus, respectively. Renal pathologist carried out histologic examinations in a blinded manner, and for each rat no less than 50 glomeruli were analysed in each kidney section.

Immunohistochemical staining for TGF-β1, PAI-1 and type IV collagen

After removal of paraffin and dehydration in xylene followed by graded alcohols, slides were immersed in distilled water and the free kidney sections were transferred to a 10 mmol/L citrate buffer solution at a pH of 6.0. Various staining conditions were then applied: sections were heated at 80°C for 30 min to retrieve antigens for TGF-β1 staining, transferred to a 1 M EDTA buffer solution (pH 8.0) for type IV collagen staining or transferred to Biogenex Retrieve (pH 8.0) (InnoGenex, San Ramon, CA, USA) and microwaved for 10–20 min for PAI-1 staining. After washing in water, 3.0% H₂O₂ in methanol was applied for 20 min in order to block endogenous peroxidase activity, and the slides were incubated at room temperature for 20 min with normal goat serum (TGF-β1) or 10% powderblock (PAI-1) to prevent nonspecific detection. Next, slides were incubated for 1 h with a primary antibody against rabbit polyclonal antitype IV collagen (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or for 2 h with a rabbit polyclonal anti-TGF-β1 antibody (1:200; Santa Cruz Biotechnology). For PAI-1 staining, slides were incubated at 4°C overnight with a rabbit anti-PAI-1 antibody (1:50; American Diagnostica, Stamford, CT, USA). Slides were incubated in a secondary antibody for 30 min. For colouration, slides were incubated at room temperature with a mixture of 0.05% 3,3′-diaminobenzidine containing 0.01% H₂O₂ and counterstained with Mayer’s haematoxylin. Negative control sections were stained under identical conditions with the buffer solution substituting for the primary antibody. In order to evaluate the results of immunohistochemical staining, glomerular fields were graded semiquantitatively. Briefly, each score reflected both changes in the extent and the intensity of staining and was graded according to a 5-point scale, with grade 0 representing very weak or absent staining and no localized increases of staining; grade 1, diffuse, weak staining with 1–25% of the glomerulus showing focally increased staining; grade 2, 25–50% of the glomerulus demonstrating focal, strong staining; grade 3, 50–75% of the glomerulus stained strongly in a focal manner and grade 4, >75% of the glomerulus stained strongly. Between 50 and 60 glomeruli were counted under high power fields (×400), and the average score was calculated.

Mesangial cell culture

A part of normal renal cortex of Sprague-Dawley rats was obtained immediately after surgical nephrectomy. Glomeruli were treated with collagenase (Gibco BRL, Gaitherburg, Md., USA) and plated on culture dishes in Dulbecco’s modified Eagle medium (DMEM, Gibco BRL) containing 17% heat inactivated fetal calf serum (Sigma Chemical, St Louis, Mo., USA). Mesangial cells were cultured in DMEM supplemented with 17% fetal calf serum, 2% of each penicillin and streptomycin, 1% HEPES, 2 g sodium bicarbonate and 2 mM l-glutamine at 37°C in 5% CO₂–95% air. Sub-confluent MCs were serum-starved for 24 h, and some wells were cultured under a high glucose condition (30 mM of d-glucose) and aldosterone (Sigma-Aldrich, St Louis, MO, USA) was added to the culture media at a final concentration of 100 nM. When the effects of enalapril and eplerenone and their combination were tested, enalaprilat (Sigma-Aldrich) that is the active form of enalapril and eplerenone and Aldexal (Sigma-Aldrich) that is the active form of Aldexal and eplerenone were added to cells at a final concentration of 2.5 µM and 10 µM, respectively, 30 min before high glucose and aldosterone treatment. All experimental groups were cultured in triplicate and harvested at 24 h for extraction of the total RNA and protein.

Measurement of secreted collagen and TGF-β1 in cultured MCs

Total soluble collagen was measured in culture supernatants by the Sircol™ soluble collagen assay kit (Bio-color, Belfast, Northern Ireland) following the manufacturer’s instructions. Briefly, 1 mL of Sirius red reagent was added to 100 µL of test samples and mixed for 60 min at room temperature in a mechanical shaker. The collagen-dye complex was precipitated by centrifugation at 14 000 g for 10 min. To release the bound dye, 1 mL of alkali reagent (0.5 M NaOH) was added to the precipitate; then the absorbance was measured at 540 nm using an ELISA reader. The amount of TGF-β1 secreted into the culture medium was quantified using a commercially available ELISA kit (R&D systems, Abingdon, Oxfordshire, UK) following the manufacturer’s instructions. Total (active plus latent fractions) TGF-β1 was measured following acid activation with
Table 1. Biochemical parameters in experimental animals

<table>
<thead>
<tr>
<th></th>
<th>LETO</th>
<th>OLETF</th>
<th>OLT+EPL(50)</th>
<th>OLT+EPL(200)</th>
<th>OLT+Ena</th>
<th>OLT+EPL+Ena</th>
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<tr>
<td>BW (g)</td>
<td>584 ± 17</td>
<td>622 ± 28</td>
<td>612 ± 13</td>
<td>587 ± 28</td>
<td>581 ± 15</td>
<td>570 ± 33</td>
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<td>KW/BW (mg/g)</td>
<td>0.29 ± 0.02</td>
<td>0.37 ± 0.05a</td>
<td>0.35 ± 0.06a</td>
<td>0.36 ± 0.02a</td>
<td>0.34 ± 0.04</td>
<td>0.33 ± 0.02</td>
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<td>FPG (mg/dL)</td>
<td>102 ± 4.2</td>
<td>133 ± 8.5b</td>
<td>124 ± 5.5b</td>
<td>135 ± 9.2b</td>
<td>123 ± 9.5b</td>
<td>126 ± 3.5b</td>
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<td>S-Cr (mg/dL)</td>
<td>0.54 ± 0.02</td>
<td>0.58 ± 0.05</td>
<td>0.57 ± 0.03</td>
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<td>0.58 ± 0.03</td>
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<td>UV (mL/day)</td>
<td>11.0 ± 4.1</td>
<td>21.5 ± 2.2a</td>
<td>16.4 ± 4.3</td>
<td>14.2 ± 6.5</td>
<td>13.1 ± 4.4</td>
<td>13.5 ± 4.8</td>
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<td>SBP (mmHg)</td>
<td>128 ± 8.4</td>
<td>141 ± 8.2a</td>
<td>134 ± 4.8</td>
<td>132 ± 6.8</td>
<td>121 ± 5.5b</td>
<td>119 ± 9.8c</td>
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<tr>
<td>P-Na (mmol)</td>
<td>143 ± 5.6</td>
<td>145 ± 7.9</td>
<td>149 ± 8.1</td>
<td>144 ± 5.7</td>
<td>147 ± 6.5</td>
<td>144 ± 5.7</td>
</tr>
<tr>
<td>P-K (mmol/L)</td>
<td>3.7 ± 0.17</td>
<td>4.12 ± 0.34</td>
<td>4.98 ± 0.38a</td>
<td>5.24 ± 0.43b</td>
<td>4.78 ± 0.45</td>
<td>5.55 ± 0.62bc</td>
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<td>UNa (mmol/day)</td>
<td>1.04 ± 0.03</td>
<td>1.21 ± 0.11</td>
<td>1.16 ± 0.27</td>
<td>1.27 ± 0.44</td>
<td>1.21 ± 0.52</td>
<td>1.31 ± 0.65</td>
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<td>UK (mmol/day)</td>
<td>2.21 ± 0.33</td>
<td>2.12 ± 0.37</td>
<td>1.85 ± 0.63</td>
<td>1.49 ± 0.54c</td>
<td>1.97 ± 0.43</td>
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<td>UK/Na ratio</td>
<td>1.92 ± 0.23</td>
<td>1.88 ± 0.27</td>
<td>1.63 ± 0.29</td>
<td>1.32 ± 0.34c</td>
<td>1.62 ± 0.49</td>
<td>1.33 ± 0.29c</td>
</tr>
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Values are expressed as mean ± SEM. OLT, OLETF; EPL, eplerenone; Ena, enalapril; BW, body weight; KW, kidney weight; FPG, fasting plasma glucose; S-Cr, serum creatinine; UV, urine volume; SBP, systolic blood pressure; P-Na, plasma sodium concentration; P-K, plasma potassium concentration; UNa, 24-h urinary sodium excretion; UK, 24-h urinary potassium excretion; UK/Na ratio, 24-h urinary ratio of potassium to sodium.

*P < 0.05, versus LETO; **P < 0.01, versus LETO; ***P < 0.05, versus OLETF.

Statistical analysis was performed using a microcomputer-assisted program with SPSS for Windows 10.0 (SPSS Inc., Chicago, IL, USA). A value of P < 0.05 was considered statistically significant.

Results

Effect of eplerenone, enalapril and their combination on biochemical parameters and urinary albumin excretion in type II diabetic rats

Table 1 shows the various biochemical results for each treatment group. Body weights were similar among the six groups. Kidney weights normalized to body weights were higher in OLETF rats than in LETO rats, and neither eplerenone nor enalapril had an effect on kidney weights. All OLETF rats, both treated and non-treated, had higher fasting plasma glucose concentrations compared with LETO rats. While serum creatinine levels were similar among all groups of rats, urine volume was significantly increased in OLETF rats. Further, the systolic blood pressures of OLETF rats were much higher than that of LETO rats. Although eplerenone treatment did not induce significant changes in systolic blood pressure even in the high-dose group, enalapril treatment including combination treatment induced significant reduction in systolic blood pressure. However, systolic blood pressure did not show significant difference between the group treated with enalapril and the group with combination treatment. Although plasma potassium levels were not different between the LETO and OLETF groups, their levels were significantly higher in OLETF rats than in LETO rats, and neither eplerenone nor enalapril had an effect on potassium levels. All OLETF rats, both treated and non-treated, had higher fasting plasma glucose concentrations compared with LETO rats. While serum creatinine levels were similar among all groups of rats, urine volume was significantly increased in OLETF rats. Further, the systolic blood pressures of OLETF rats were much higher than that of LETO rats. Although eplerenone treatment did not induce significant changes in systolic blood pressure even in the high-dose group, enalapril treatment including combination treatment induced significant reduction in systolic blood pressure. However, systolic blood pressure did not show significant difference between the group treated with enalapril and the group with combination treatment. Although plasma potassium levels were not different between the LETO and OLETF groups, their levels were significantly higher in OLETF rats treated with eplerenone and groups with combination treatment, whereas plasma sodium concentrations were the same among all groups. These changes in plasma potassium levels were associated with decreased urinary potassium excretion in the eplerenone treatment groups including the combination group.

Urinary albumin excretion (UAE) in OLETF rats was significantly higher than that in LETO rats, and it was dose-dependently decreased in the groups with eplerenone treatment. Compared with eplerenone, enalapril treatment
Effect of eplerenone, enalapril and their combination treatment

Figure 1. Effects of eplerenone and enalapril on urinary albumin excretion and creatinine clearance. (A) Twenty-four-hour urinary albumin excretion corrected by urine creatinine concentration. (B) Creatinine clearance calculated on the basis of plasma and urinary creatinine concentrations and the corresponding urine volume and is expressed as millilitres per minute per 100 grams body weight. Data are shown as mean ± SEM. EPL, eplerenone; Ena, enalapril; *P < 0.05, versus LETO; **P < 0.01, versus LETO; ***P < 0.001, versus LETO; #P < 0.05, versus OLETF; ##P < 0.01, versus OLETF; ###P < 0.001, versus OLETF; θP < 0.05, versus OLETF with eplerenone 200 and P < 0.01, versus OLETF with eplerenone 50.

showed more reduced UAE. Interestingly, there was an additive decrease in UAE in the combination group (Figure 1A). In addition, creatinine clearance was averaged 0.56 ± 0.14 mL/min/100 g body weight in LETO rats and was significantly lower in OLETF rats (0.23 ± 0.04 mL/min/100 g body weight) at the end of the study period (Figure 1B). Although low dose of eplerenone group did not restore their levels to control LETO rats, high dose of eplerenone treatment significantly increased creatinine clearance to near control LETO rats. However, enalapril treatment including combination treatment did not show significant changes in creatinine clearance compared with untreated OLETF groups (Figure 1B).

Effect of eplerenone, enalapril and their combination on renal histologic changes in type II diabetic rats

Figure 2 shows the histologic findings for the control LETO rats (Figure 2A,G,M,S), diabetic OLETF rats (Figure 2B,H,N,T) and OLETF rats treated with low dose of eplerenone (Figure 2C,I,O,U) and high dose of eplerenone (Figure 2D,J,P,V), enalapril (Figure 2E,K,Q,W) and the combination of eplerenone and enalapril (Figure 2F,L,R,X). Diabetic OLETF rats displayed severe glomerulosclerosis compared with control LETO rats (Figure 2A,B), and while eplerenone or enalapril treatment alone significantly improved the status of glomerulosclerosis, a combination of both drugs produced even greater benefits (Figure 2A,B). In eplerenone groups, glomerular pathology was dose-dependently improved. However, tubulointerstitial changes were not remarkable even in OLETF rats, so significant differences were not found among the experimental groups (Figure 2A–F).

We next examined the immunohistochemical staining of transforming growth factor β1 (TGF-β1), type IV collagen and plasminogen activator inhibitor-1 (PAI-1) in the kidney (Figure 2G–X). In the diabetic kidney, TGF-β1 and type IV collagen immunoreactivity was markedly increased in glomerular mesangium, although this was not accompanied by a significant increase in tubulointerstitial area compared with that of control LETO rats (Figure 2G–R). For PAI-1 staining, the results were more impressive. PAI-1 deposition was hardly observed in the kidneys of control LETO rats; however, diabetic OLETF rat kidneys dramatically exhibited strong positive PAI-1 staining detected mainly in glomeruli (Figure 2S–X). Eplerenone or enalapril treatment induced significant reductions in renal TGF-β1, PAI-1 and type IV collagen protein expressions, in accordance with the degree of glomerulosclerosis. It was of interest that the combination of both eplerenone and enalapril further reduced expressions of TGF-β1, type IV collagen and PAI-1 protein in diabetic OLETF rats beyond what was observed with either of the two treatments alone (Figure 2G–X).

Figure 3 shows the quantitative scoring index of glomerulosclerosis and immunohistochemical staining of TGF-β1, type IV collagen and PAI-1, respectively. The glomerulosclerosis index was markedly increased in OLETF rats compared with LETO rats, and it was dose-dependently decreased in eplerenone groups. As shown in Figure 3A, glomerulosclerosis was significantly improved in the enalapril group, and most dramatic improvement was observed in the combination group (LETO, 0.02 ± 0.003; OLETF, 2.35 ± 0.35; OLETF with low dose eplerenone, 1.83 ± 0.31; OLETF with high dose eplerenone, 1.23 ± 0.31; OLETF with enalapril, 0.78 ± 0.10; OLETF with eplerenone plus enalapril, 0.34 ± 0.06). In OLETF rats treated with eplerenone or enalapril alone, the scoring
indices of TGF-β₁, type IV collagen and PAI-1 were significantly decreased, whereas the most dramatic decrease was observed in the combination group (TGF-β₁, LETO, 0.21 ± 0.06; OLETF, 2.31 ± 0.10; OLETF with low dose of eplerenone, 1.93 ± 0.21; OLETF with high dose of eplerenone, 1.20 ± 0.31; OLETF with enalapril, 0.91 ± 0.10; OLETF with eplerenone plus enalapril, 0.34 ± 0.08; Type IV collagen, LETO, 0.52 ± 0.11; OLETF, 3.12 ± 0.10; OLETF with low dose of eplerenone, 2.56 ± 0.59; OLETF with high dose of eplerenone, 1.86 ± 0.29; OLETF with enalapril, 1.21 ± 0.27; OLETF with eplerenone plus enalapril, 0.72 ± 0.14; PAI-1, LETO, 0.09 ± 0.02; OLETF, 2.43 ± 0.44; OLETF with low dose of eplerenone, 1.80 ± 0.43; OLETF with high dose of eplerenone, 1.21 ± 0.23; OLETF with enalapril, 0.88 ± 0.21; OLETF with eplerenone plus enalapril, 0.33 ± 0.11).

To evaluate the gene expressions of TGF-β₁, type IV collagen and PAI-1, we performed real-time PCR using tissues isolated from rat kidneys. In accordance with the findings from immunohistochemical staining of the rat kidneys, TGF-β₁ gene expression was significantly higher in OLETF rats compared with control LETO rats. Similarly, gene expressions of type IV collagen and PAI-1 were higher in OLETF rats. On their own, eplerenone or enalapril inhibited gene expressions of TGF-β₁, type IV collagen and PAI-1, and a combination of these two drugs suppressed the expressions even more dramatically (Figure 4).

Effect of eplerenone, enalapril and their combination on the serum and urinary excretion of βig-h3 in type II diabetic rats

We measured serum and urinary βig-h3, which has been used to assess the biological activity of TGF-β₁ in various tissues, including the kidney [19]. As demonstrated in Figure 5, the urinary βig-h3 concentration was nine times higher in diabetic OLETF rats than in control LETO rats. The effect of eplerenone and enalapril on the excretion of urinary βig-h3 was consistent with the aforementioned results. Significantly, treatment with either eplerenone or enalapril alone led to reduced urinary βig-h3 excretion in diabetic OLETF rats, more dramatically so in the combination group. There were no differences in serum βig-h3 concentrations among groups with OLETF rats with or without treatment.

Effect of eplerenone, enalaprilat and their combination on the synthesis of TGFβ₁, type IV collagen and PAI-1 in cultured mesangial cells

To determine the direct effect of eplerenone, enalapril and their combination on the expressions of high glucose and aldosterone-induced profibrotic and proinflammatory molecules in intrinsic renal cells, we performed a real-time quantitative analysis of TGF-β₁, type IV collagen
and PAI-1 mRNA expressions in mesangial cells. Although high glucose treatment increased type IV collagen and TGF-β1 gene expressions, PAI-1 expression was not increased significantly under high glucose stimuli. However, administration of aldosterone under a high glucose condition induced a marked up-regulation of PAI-1 gene expression (Figure 6). Furthermore, aldosterone administration induced a further increase in type IV collagen and TGF-β1 gene expressions under a high glucose condition. Prior treatment of eplerenone or enalaprilat significantly reduced gene expressions of TGF-β1, type IV collagen and PAI-1 induced by high glucose and aldosterone stimulation. Interestingly, combined treatment with eplerenone and enalaprilat induced a further decrease in TGF-β1, type IV collagen and PAI-1 gene expressions compared with eplerenone or enalaprilat treatment alone (Figure 6). Next, we measured gene expression in only high glucose-stimulated MCs without aldosterone stimulation to exclude the effect of aldosterone on TGF-β1, type IV collagen and PAI-1 mRNA expressions. As shown in Figure 7, high glucose-induced TGF-β1 and type IV collagen expressions were slightly decreased by eplerenone and enalaprilat treatment, although not significant. Combination treatment in MCs also showed the most profound effect in high glucose-induced TGF-β1, type IV collagen expressions. However, there were no changes in PAI-1 expression in only high glucose-stimulated MCs (Figure 7).

We further evaluated whether gene expressions of TGF-β1, type IV collagen and PAI-1 were related to their protein production. As shown in Figure 8A–B, secretory protein levels for TGF-β1, and total soluble collagen were significantly increased by high glucose and aldosterone stimulation, which was significantly inhibited by eplerenone or enalaprilat treatment. In agreement with gene expression, combined treatment with eplerenone and enalaprilat almost completely abolished high glucose and aldosterone-induced TGF-β1 and collagen protein secretion. In addition, PAI-1 protein synthesis evaluated by western blot also showed that the most dramatic decrease was observed in the combined treatment group (Figure 8C).

**Discussion**

In the present study, we firstly demonstrated the additive beneficial effects of combined blockade of both eplerenone and enalapril in a type II diabetic rat model. The renoprotective effect of aldosterone inhibition has been found in both animal models of renal disease [8–12,20–24] and human clinical trials [14–16,25–27]. Here, we extended our
Aldosterone blockade treatment in addition to ACE inhibition additively decreased UAE and improved the status of glomerulosclerosis, suggesting that the inhibition of the aldosterone system has additional beneficial effects independent of renin–angiotensin blockade on diabetic nephropathy. The effects of combined aldosterone and RAS blockade have been recently observed in experimental models of hypertensive kidney disease [30,31], radiation-injured renal injury [24] and proteinuric kidney disease [32]; however, prior to this study, no similar analysis has been performed in an animal model of diabetic nephropathy.

Although there have been many clinical studies on the effect of aldosterone blockade in diabetic patients [14–16,26,27,33–36], the exact mechanisms responsible for the beneficial effects of aldosterone inhibition on diabetic nephropathy are still unclear. In this study, we found that eplerenone treatment dose dependently decreased the expressions of type IV collagen and TGF-β1, both of which are linked to the development of glomerulosclerosis in diabetic nephropathy. Although we were unable to provide direct evidence that aldosterone induced TGF-β1 up-regulation or that aldosterone synthesis is up-regulated in the diabetic kidney, recent studies suggest the possibility that aldosterone provokes renal TGF-β1 synthesis [37] and

previous studies [28,29] by analysing the effect of aldosterone blockade in addition to ACE inhibition in an animal model of type II diabetic nephropathy.
that aldosterone synthesis is up-regulated in the diabetic kidney [38].

Furthermore, we also found that eplerenone and enalapril treatment profoundly suppressed the expression of PAI-1 in the diabetic kidney, and mitigation of the effects of PAI-1 by eplerenone suggests that aldosterone may contribute to overproduction of PAI-1 in the diabetic kidney. Interestingly, PAI-1 expression was more drastically ameliorated by eplerenone treatment when compared with its effect on TGF-β1 expression. This result agrees with recent studies that show that aldosterone directly increases PAI-1 synthesis in mesangial cells [39] and spironolactone therapy decreases the plasma level of PAI-1 in patients with diabetic nephropathy [40].

In this study, we observed that eplerenone or enalapril treatment significantly decreased UAE but had no effect on blood glucose levels, body weight or kidney to body weight ratios. Systolic blood pressure was not significantly reduced by eplerenone therapy alone even in the high-dose group, whereas enalapril treatment and a combination of these two therapies significantly lowered systolic blood pressure, suggesting that the beneficial effect of eplerenone therapy is independent of both haemodynamic and metabolic mechanisms, and that an additional haemodynamic mechanism may contribute to a beneficial effect in the enalapril group and in the combination group. However, systolic blood pressure between the enalapril group and the combination group was similar, which suggests that non-haemodynamic mechanisms rather than haemodynamic factors may result in the additive beneficial effect of the combination therapy. In this study, we observed that high dose of eplerenone treatment significantly increased creatinine clearance and the enalapril treatment group and the combination group did not show significant changes in creatinine clearance, whereas they showed histological improvement. This discrepancy might have been caused by several factors. Firstly, the enalapril treatment group and the combination group showed significant reduction in systolic blood pressure, which might have reduced renal perfusion pressure that resulted in decreased creatinine clearance. The second possibility is that the effect of enalapril on glomerular haemodynamics such as the preferential effect of enalapril on efferent arteriole might have led to decreased creatinine clearance. Inhibition of the RAS by either an ARB or an ACEI have been reported to induce a reversible reduction in intraglomerular pressure in most nephrons [41]. In the case of defect in autoregulatory capacity for renal blood flow such as diabetes mellitus, creatinine clearance will be more profoundly decreased when RAS activity is blocked under a low systolic blood pressure condition [41]. In this study, we observed that high dose of eplerenone treatment significantly increased creatinine clearance and the enalapril treatment group and the combination group did not show significant changes in creatinine clearance, whereas they showed histological improvement.
treatment further aggravated hyperkalaemia. These results indicate that one should be cautious in deciding to treat patients with combination regimen containing aldosterone receptor antagonist and RAS inhibitor.

To define the molecular mechanisms of the additive beneficial effect of combination therapy and to exclude the effect of haemodynamic mechanism in the combination therapy, we performed an in vitro study. The above-mentioned in vivo additive renal protective effects of combination therapy are further supported by the in vitro experiments that demonstrated an additive inhibitory effect of combination of eplerenone and enalaprilat on the synthesis of TGF-β1, type IV collagen and PAI-1 induced by high glucose and aldosterone treatment in cultured mesangial cells. Although we did not test the beneficial effects in other glomerular cells such as podocyte and endothelial cells, our findings in mesangial cells suggest that the direct cellular effect of combination treatment may contribute to the observed in vivo additive beneficial effects in our study.

In the present study, we also demonstrated the effect of eplerenone and enalapril on urinary βig-h3 excretion, which is a biologic marker associated with the activation of TGF-β1 [19]. Although there was no significant difference in serum βig-h3 levels after treatment, urinary excretion of βig-h3, which reflects the activity of TGF-β1 in the kidney, was dramatically increased in the diabetic kidney, and while both eplerenone and enalapril alone were able to markedly decrease urinary excretion of βig-h3, treatment with both drugs led to a more profound decrease. These results were in accordance with the results of gene expression and immunohistochemical results for TGF-β1.

During the long-term use of RAS blockade, which includes ACE inhibitors or angiotensin II receptor antagonists or a combination of both, plasma aldosterone levels have been shown to increase in some patients (aldosterone escape phenomenon). Furthermore, the aldosterone escape during long-term blockade of the RAS system is associated with an enhanced decline in GFR in patients with type I diabetic patients with nephropathy [42]. The data from the present study imply that aldosterone inhibition based on RAS blockade may provide more complete interruption of RAAS, thereby resulting in enhanced renal protective effects. Indeed, recent clinical trials have demonstrated that add-on therapy with spironolactone based on RAS inhibition provides more anti-proteinuric effects in patients with diabetic nephropathy [30,31,35].

In conclusion, this study suggests that the combination of aldosterone blockade with RAS inhibition is more effective in preventing diabetic renal damage than either therapy alone. The combined treatment approach was associated
with a reduction of renal production of profibrotic cytokines such as TGF-β1, PAI-1 and type IV collagen. Taken together, these results indicate that blockade of the aldosterone system based on RAS inhibition may be a new therapeutic strategy for retarding the progression of diabetic nephropathy.

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References


33. van den Meiracker AH, Baggen RG, Pauli S. Spironolactone in type 2 diabetic nephropathy: effects on proteinuria, blood pressure and renal function. *J Hypertens* 2006; 24: 2285–2292


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