Combination therapy with an angiotensin II receptor blocker and an HMG-CoA reductase inhibitor in experimental subtotal nephrectomy

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Abstract

Background. Angiotensin receptor 1 blockers (ARB) are standard nephroprotective drugs in chronic kidney disease. There is less evidence for a nephroprotective effect of HMG-CoA reductase inhibitors (statins) and much less is known about potential benefits of combination therapy. We evaluated the therapeutic potential of a statin alone or in combination with an ARB in experimental chronic kidney disease.

Methods. Subtotally nephrectomized (5/6 Nx) rats were treated early with vehicle, losartan, cerivastatin or losartan/cerivastatin. Expression of messenger RNA (mRNA) was assessed by real-time reverse transcription–polymerase chain reaction. Tissue proteins were localized by immunohistochemistry. Nuclear factor-κB (NF-κB) activation was measured in whole kidneys.

Results. In contrast to the sham group, at 6 weeks, vehicle-treated 5/6 Nx rats displayed renal lesions, albuminuria and increased serum creatinine and total kidney NF-κB p65 DNA-binding activity and preproendothelin-1, fibronectin and type I and III collagen mRNA. NF-κB activation correlated with albuminuria and histological renal injury. Losartan or combination therapy preserved renal function, abrogated albuminuria and improved glomerular and interstitial histology. Cerivastatin alone preserved renal function and improved interstitial injury but did not influence albuminuria, glomerular histology or NF-κB activation. Losartan/cerivastatin normalized kidney NF-κB activation and extracellular matrix mRNA expression pattern. The effect of losartan alone on these parameters was less intense. All treatments decreased preproendothelin-1 mRNA and preserved interstitial capillaries.

Conclusions. In a chronic kidney disease model, early treatment with either an ARB or a statin preserved renal function although the mechanisms differed. Combination therapy with an ARB and a statin did not confer clear-cut advantages on biochemical and histological parameters over ARB alone, although it further improved the kidney NF-κB and gene expression profile.

Keywords: angiotensin II receptor blocker; HMG-CoA reductase inhibitor; NF-κB; subtotal nephrectomy

Introduction

The remnant kidney model of 5/6 nephrectomy (5/6 Nx) in rats is often used to study the mechanisms of and potential therapeutic approaches to progression of chronic kidney disease (CKD) with renal mass reduction [1–3]. In this model, systemic hypertension and proteinuria contribute to kidney injury and to the expression of pro-inflammatory and pro-fibrotic molecules by kidney cells [4–6]. Angiotensin II is involved in the pathogenesis of renal disease [7]. Indeed, angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARBs) have anti-hypertensive and anti-proteinuric actions and currently are the mainstay of therapy to retard progression of CKD in clinical practice [8]. ARBs and ACEIs have been successfully used to treat rats with remnant kidneys [9]. Although they are quite effective in decreasing blood pressure and albuminuria, and in reducing the rate of glomerular filtration rate (GFR) decline, they do not seem to be able to stabilize or improve GFR by themselves [10]. Thus, novel therapeutic strategies are needed that may involve combination with other agents. The physiopathological actions of angiotensin II are partly attributable to nuclear factor-κB (NF-κB) activation [11]. This transcription factor has been associated with progressive renal injury [12, 13]. NF-κB regulates the expression of key genes involved in kidney injury [12, 14, 15]. The main heterodimer of the canonical NF-κB pathway is composed of p50 and the DNA-binding proteins p65/RelA [12].

Statins or 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors lower serum cholesterol levels and prevent cardiovascular events [16]. Statins are frequently prescribed to kidney patients and some data point to a nephroprotective effect, as recently reviewed [17]. However, there is some controversy in this regard. Thus, a meta-analysis concluded that
statins may have a beneficial effect on pathological albuminuria [18]. However, data from the Prevention of REnal and Vascular ENd-stage Disease Intervention trial (PREVEND-IT) and the PREVEND cohort study indicated that statins do not lower albuminuria in subjects selected because of an elevated albuminuria either in the presence or absence of an ACEI [19]. However, there is recent evidence that proteinuria and nephroprotection may be dissociated [20]. In this regard, more research into the mechanisms and magnitude of potential statin nephroprotection is needed. In this regard, the use of cerivastatin is associated with decreased plasma and urinary endothelin-1 levels in microalbuminuric patients with type 2 diabetic mellitus [21]. In animal models, cerivastatin reduced angiotensin II-induced albuminuria and systolic blood pressure (SBP) [22] and also reduced proteinuria and renal fibrosis independently of blood pressure in stroke-prone spontaneously hypertensive rats [23]. However, there is little information on statins and renal mass reduction and on the potential cooperation with ARBs.

This study was designed to evaluate the therapeutic effect on biochemical parameters, kidney histology, NF-κB activation and gene expression of the statin cerivastatin alone or in combination with the ARB losartan [7, 24] in experimental CKD caused by renal mass reduction. Cerivastatin preserved renal function and improved interstitial injury. The combination therapy with cerivastatin and losartan was not shown to have a clear advantage on renal function, albuminuria or histology over losartan alone, although it led to a more favorable pattern of renal gene expression than either drug alone.

Methods

Animals

Male Sprague–Dawley rats weighing 175 to 225 g were studied. Rats were kept under a 12/12 h light/dark cycle with free access to tap water and fed with standard laboratory rat chow containing 17% protein and 0.25% sodium. Animals were handled following the Spanish guidelines for care of laboratory animals (RD 1201/2005; LEY 32/2007).

Experimental studies

Animals were subjected to two-stage 5/6 Nx consisting of ablation of the right kidney, followed one week later by ligation of the renal artery branches irrigating two-thirds of the left kidney as previously described [25]. After surgery, rats were randomly assigned to different therapies: (i) losartan \( n = 8; 200 \text{ mg/L in the drinking water; Merck Sharp & Dohme, Spain} \); (ii) cerivastatin \( n = 7; 0.1 \text{ mg/kg/day by oral gavage; Bayer AG, Wuppertal, Germany} \) and (iii) a combination of losartan plus cerivastatin \( n = 9 \). Treatment started one week after the 5/6 Nx (Day 7) and continued until the end of the study (Day 42). Drug doses were chosen on the basis of previous studies [7, 26]. A group of 5/6 Nx rats \( n = 8 \) treated with vehicle served as control. A sham group \( n = 11 \) underwent a ventral laparotomy under anesthesia but only handled the renal pedicle without removing the renal mass.

SBP and 24-h albuminuria were measured the day of right kidney removal (Day –7), at the onset of treatment (Day 7), 24 days after 5/6 Nx, and at the end of the study. SBP was measured by the tail-cuff plethysmography method (Letica Scientific Instruments, Barcelona, Spain), as previously reported [17, 27–33]. Albuminuria was measured in 24-h urine samples (mg/day) by an immunoturbidimetric test (Roche Diagnostics GmbH, Mannheim, Germany).

Rats were sacrificed under anesthesia, blood was collected, and an intact part of the perfused kidney was obtained to perform molecular and morphological analysis. Serum creatinine was measured by an enzymatic colorimetric test (Boehringer-Mannheim, Mannheim, Germany).

In set-up experiments, rats were found at Day 7 post 5/6 Nx to be hypertensive and proteinuric (Figure 1A and B). In addition, they had increased serum creatinine and increased kidney expression of genes coding for extracellular matrix genes such as type I collagen and fibronectin (Supplementary figure 1).

Morphology and Immunohistochemistry

Renal scarring was studied on paraffin-embedded 5-μm-thick transverse sections of kidney stained with Masson’s trichrome and periodic Schiff reagent haematoxulin (PASH). Nine fields per kidney sample were randomly selected. Glomerulosclerosis (Glomerulosclerosis Index score: GIS) was semiquantitatively scored using the sections stained with periodic acid–Schiff. Each glomerulus was graded from 1 to 4: grade 1, normal glomerulus by light microscopy; grade 2, involvement of up to one-third of the glomerular area; grade 3, involvement of one- to two-thirds of the glomerulus; and grade 4, two-thirds of global sclerosis. Each score was then calculated according to the formula GIS = \([1 \times \text{number of grade 2 glomeruli} + (2 \times \text{number of grade 3 glomeruli}) + (3 \times \text{number of grade 4 glomeruli})] \times 100/\text{(number of glomeruli observed)} \) [34]. The tubulointerstitial lesions in the cortex was graded as follows: 0, normal; 1, area of interstitial inflammation and fibrosis, tubular atrophy, and dilation with cast formation involving <25% of the field; 2, lesion area between 25% and 50% of the field; and 3, lesions involving >50% of the field [35]. All parameters were blindly analysed by a single observer.
Interstitial fibrosis was stained with Sirius red, which stains extracellular matrix, mainly collagens [36, 37].

Tissue localization of selected proteins was assessed by immunohistochemistry using the standard streptavidin-biotin peroxidase method in serial sections (5 μm thick). Primary antibodies were: 1:200 mouse monoclonal anti-NF-κB p65 (SC-8008, Santa Cruz); 1:100 rabbit polyclonal antiendothelin-1 (6901, Peninsula); 1:200 biotinylated goat antimonoclonal antifibrinogen (AB2033, Millipore, MA); 1:300 mouse anti-aminopeptidase-P (BMS1104, Ebioscience) for peritubular capillaries and anti-CD68 (IS613, Dako, Glostrup, Denmark) for macrophages; 1:200 biotinylated goat antimouse or antirabbit (Caltag) goat antimouse or antirabbit (Caltag) were used as secondary antibodies, followed by 1:200 streptavidin/biotin horseradish peroxidase (Caltag) and diaminobenzidine visualization. Sirius red and CD68 stainings were followed by 1:200 streptavidin/biotin horseradish peroxidase (Caltag) and diaminobenzidine visualization. Sirius red and CD68 stainings were followed by 1:200 streptavidin/biotin horseradish peroxidase (Caltag) and diaminobenzidine visualization. Sirius red and CD68 stainings were followed by 1:200 streptavidin/biotin horseradish peroxidase (Caltag) and diaminobenzidine visualization. Sirius red and CD68 stainings were followed by 1:200 streptavidin/biotin horseradish peroxidase (Caltag) and diaminobenzidine visualization.

Results

Losartan or cerivastatin preserve renal function but only losartan reduces albuminuria and SBP

Albuminuria, serum creatinine and SBP increased in 5/6 Nx animals (Table 2 and Figure 1). Just before treatment initiation, 5/6 Nx rats had increased SBP compared to sham animals (Figure 1A). SBP was reduced in 5/6 Nx rats receiving losartan or the combination of losartan plus cerivastatin from Day 24, with normalization at Day 42. While SBP progressively increased in control 5/6 Nx rats during follow-up, cerivastatin alone prevented the magnification of SBP after Day 7 of the experiment.

All 5/6 Nx rats showed albuminuria before the start of treatment (Figure 1B). By the end of the experiment, losartan plus cerivastatin or losartan alone reversed albuminuria, in contrast with the lack of effect of cerivastatin alone (Table 2 and Figure 1B).

Losartan alone, cerivastatin alone or the losartan plus cerivastatin combination prevented the decrease in renal function, as evaluated by serum creatinine (Table 2).

Losartan alone or in combination with cerivastatin improved glomerular and tubulointerstitial injury while cerivastatin alone improved tubulointerstitial injury

The 5/6 Nx rats evidenced glomerular injury characterized by mesangial matrix expansion, hyalinosis, mesangiolysis, capillary dilatation, intracapillary thrombosis, adhesion of the glomerular tuft to Bowman’s capsule or glomerular necrosis (Figure 2). Evidence of tubulointerstitial injury included focal tubular atrophy, tubular dilatation, cast formation, thickening of tubular basement membrane, and interstitial fibrosis with enlargement of interstitial space (Figure 2).

Treatment with losartan alone or in combination with cerivastatin prevented glomerular injury (Table 2, Figure 2) following renal ablation. There was a non-significant trend towards better preservation of glomerular morphology in rats treated with cerivastatin alone correlations were used to determine the association of variables. \( P < 0.05 \) indicated statistical significance.

Table 1. Primer sequences and conditions of PCR used in the LightCycler Instrument

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Accession number (GenBank)</th>
<th>Primer sequences (forward and reverse)</th>
<th>MgCl₂ (mM)</th>
<th>Amplification program</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin 1</td>
<td>NM_019143</td>
<td>GGCTGCAGCTTCAAATCCCTTCT; AGTCTCTTACGCCCTCAAT</td>
<td>2.5</td>
<td>40× (95°C, 10 sec; 58°C, 5 sec; 72°C, 10 sec)</td>
</tr>
<tr>
<td>Preproendothelin-1</td>
<td>NM_025448</td>
<td>GTGTCCTCAGGTCGCGAATGTTGTT; GCAGGAGAGCAGGAGATTTG</td>
<td>2.5</td>
<td>42× (95°C, 10 sec; 58°C, 1 sec; 72°C, 27 sec)</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>NM_053304</td>
<td>GTGTCCTCGGTTGCGACGATTTG; GCCGTCGCTGGTCTGGTT; GCCGTCGCTGGTCTGGTT</td>
<td>3.0</td>
<td>50× (95°C, 5 sec; 58°C, 5 sec; 72°C, 8 sec)</td>
</tr>
<tr>
<td>Type III collagen</td>
<td>NM_032085</td>
<td>GCCGTCGCTGGTCTGGTT; GCCGTCGCTGGTCTGGTT; GCCGTCGCTGGTCTGGTT</td>
<td>3.0</td>
<td>50× (95°C, 5 sec; 58°C, 5 sec; 72°C, 8 sec)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>BC059110</td>
<td>TCCCTGAGTGGTGCCGAGAGATCCAGAGTCCTCAAGATGGTGCCAGACGAGATCCAGAGCAGCAT</td>
<td>2.5</td>
<td>35× (95°C, 10 sec; 58°C, 5 sec; 72°C, 13 sec)</td>
</tr>
</tbody>
</table>

*Samples were analysed in three independent PCR experiments. The specificity of the amplified products was verified by melting curve analysis immediately after the amplification program and by automated sequencing (ABI PRISM® 310 Genetic Analyzer, Applied Biosystems, Foster City, CA).*
Table 2. Albuminuria, serum creatinine and renal morphologic parameters in 5/6 Nx and sham rats at the end of the study

<table>
<thead>
<tr>
<th>Albuminuria (mg/day)</th>
<th>Serum creatinine (mg/dL)</th>
<th>GIS</th>
<th>TIL score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sham</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 ± 0.4</td>
<td>0.52 ± 0.05</td>
<td>10 ± 2</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td><strong>5/6 Nx</strong> Control</td>
<td>30 ± 4</td>
<td>14 ± 0.02</td>
<td>138 ± 19</td>
</tr>
<tr>
<td><strong>5/6 Nx Losartan</strong></td>
<td>2.0 ± 0.4</td>
<td>0.96 ± 0.07</td>
<td>40 ± 12</td>
</tr>
<tr>
<td><strong>5/6 Nx Cerivastatin</strong></td>
<td>33 ± 11</td>
<td>0.87 ± 0.03</td>
<td>96 ± 22</td>
</tr>
<tr>
<td><strong>5/6 Nx L + C</strong></td>
<td>1.1 ± 0.5</td>
<td>0.89 ± 0.03</td>
<td>29 ± 6</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM. L + C, losartan plus cerivastatin. GIS: Glomerulosclerosis index score, TIL: tubulointerstitial lesions.

*P < 0.01 versus sham.

Combination therapy with an angiotensin II receptor blocker and a statin

Fig. 2. Effect of treatment on histological parameters. (A) Losartan alone or in combination with cerivastatin improved glomerular and tubulointerstitial injury, while cerivastatin alone improved tubulointerstitial injury. Morphology of renal tissue 42 days after 5/6 nephrectomy. Normal histology in sham rats (a and b). Subtotally nephrectomized non-treated rats (control, c-f) showed capillary dilations and thrombosis (c), matrix mesangial deposits (c and d), hyalinosis (d, top), thicker Bowman’s capsules (d, bottom), interstitial infiltrate (e and f) with isolated macrophages (c, detail), and dilated tubuli containing PAS-positive cellular detritus (e, bottom; f, top), surrounded with PAS-positive thicker basal laminas (e and f). These pathological changes contrast with normal histology from losartan + cerivastatin (k and l) and losartan-treated animals (g and h), although PAS-positive material remains slightly increased in rats given losartan alone (h). Cerivastatin-treated animals (i and j) showed tubulointerstitial injury improvement. Masson trichrome stain (left) and PAS stain (right). Original magnification ×200.

(B) All treatments improve interstitial fibrosis and inflammation. Both CD68 (left) and Sirius red (right) staining confirmed significant improvement in tubulointerstitial injury in all treatment groups. Interstitial macrophages, stained with anti-CD68, and the interstitial area occupied by extracellular matrix, mainly collagen, stained with Sirius red, were quantified manually and using Image Pro Plus 4.5 software, respectively. Sh, sham; Ct, 5/6Nx control; Los, losartan; Cer, cerivastatin; L + C, losartan plus cerivastatin. *P < 0.01 versus sham. #P < 0.05 versus 5/6Nx control. CD68 stain (left) and Red Sirius stain (right). Original magnification ×200.
Table 2). Tubulointerstitial injury scores were remarkably attenuated in rats given losartan or losartan plus cerivastatin as compared with control remnant kidneys (Table 2, Figure 2A). Cerivastatin also significantly decreased tubulointerstitial injury, but to a lesser extent than regimes containing losartan (Table 2). Quantitation of extracellular...
matrix deposition by Sirius red staining and of infiltrating macrophages also disclosed significant improvements in interstitial fibrosis and inflammation in all treatment groups (Figure 2B).

The losartan-cerivastatin combination decreases total kidney NF-κB p65 DNA-binding activity in 5/6 Nx rats

Total kidney NF-κB p65 DNA-binding activity was increased in control 5/6 Nx rats (Figure 3A). Immunohistochemistry localized nuclear p65 staining in glomerular, tubular and interstitial cells of control 5/6 Nx rats (Figure 3B) but not in sham animals (Figure 3B). There was a trend toward decreased NF-κB p65 DNA-binding activity in rats treated with losartan (Figure 3A), but a significant decrement was only observed in rats receiving both losartan and cerivastatin (Figure 3A). Consistently, nuclear p65 immunoreactivity was negative in kidneys from rats treated with the combination of losartan plus cerivastatin (Figure 3B). Losartan alone did not prevent glomerular p65 staining (Figure 3B), while tubular and interstitial reaction was scarce. In contrast, cerivastatin did not modify total kidney NF-κB p65 DNA-binding activity (Figure 3A) and kidneys from animals treated with this drug showed positive nuclear p65 immunoreactivity (Figure 3B).

In rats not receiving pharmacological agents, total kidney NF-κB p65 DNA-binding activity correlated with albuminuria (Figure 4A), histological glomerular injury (GIS; Figure 4B) and histological tubulointerstitial injury (TIL; Figure 4C).

The losartan-cerivastatin combination normalizes total kidney expression of extracellular matrix components fibronectin 1 and type I and III collagen mRNA expression

Total kidney fibronectin 1 (Figure 5A), and type I and III collagen (Figure 6A) mRNA levels were clearly higher in control 5/6 Nx rats than in sham animals. Fibronectin was constitutively expressed in glomerular and tubular basement membranes in sham animals (Figure 5B). Following subtotal nephrectomy, fibronectin 1 expression increased in glomeruli and in the tubulointerstitial space (Figure 5B).
Total remnant kidney fibronectin 1, and type I and III collagen transcript levels in rats given losartan plus cerivastatin resembled sham values (Figures 5A and 6A). Immunohistochemistry confirmed decreased fibronectin 1 and type I collagen staining in rats with combination therapy (Figures 5B and 6B). The upregulation of fibronectin and type I and III collagen mRNA was not completely reversed by losartan or cerivastatin alone (Figures 5A and 6A).

The losartan-cerivastatin combination or either agent alone normalize total kidney pre-proendothelin-1 mRNA expression

Total kidney pre-proendothelin-1 mRNA levels were increased in control 5/6 Nx rats compared to sham animals (Figure 7A). Endothelin-1 expression was very scarce in the sham group (Figure 7B) and intense in control remnant kidneys (Figure 7B). Total remnant kidney pre-proendothelin-1 transcript levels in rats given losartan plus cerivastatin resembled sham values (Figure 7A). Immunohistochemistry confirmed decreased endothelin-1 staining in rats with combination therapy (Figure 7B). Losartan or cerivastatin alone also normalized the expression of pre-proendothelin-1 (Figure 7A and B).

Losartan, cerivastatin, and their combination preserve the interstitial capillary network in 5/6 Nx rats

Loss of peritubular capillaries is frequently associated with tubulointerstitial injury and correlated with interstitial fibrosis [41]. The mean number of aminopeptidase P positive capillaries was reduced in control 5/6 Nx, but not in treated animals (Figure 8).

Discussion

The main finding of the study relates to the effect of early treatment with cerivastatin alone or the losartan-
cerivastatin combination on biochemical (serum creatinine, albuminuria), histological parameters of renal injury and renal NF-κB activation and gene expression when compared to either drug alone in the subtotal nephrectomy model of renal mass reduction. The losartan-cerivastatin combination did not improve biochemical or histological renal injury over the use of losartan alone. This lack of significant additional efficacy may be in part due to the degree of improvement obtained with losartan alone. Thus, in losartan-treated animals, albuminuria and histological score did not differ from sham rats. However, combination therapy failed to significantly improve parameters not normalized by losartan alone, such as serum creatinine and glomerular histological injury. Despite this lack of benefit over hard end-points, the treatment combination achieved significant improvements in renal NF-κB activation or the renal expression of genes encoding extracellular matrix proteins when only a trend was observed for losartan alone. Thus, a benefit of the combination under certain experimental or clinical conditions cannot be excluded. Another significant finding of the study is the protection offered by cerivastatin alone on key outcome parameters, such as serum creatinine and histological tubulointerstitial injury, and the fact that this occurred despite lack of effect on albuminuria or total kidney NF-κB activity. This observation adds to mounting evidence that the response of proteinuria and renal function to therapy may be dissociated. The fact that despite a more favourable profile on a number of molecular parameters, the combination does not improve serum creatinine, is a striking finding of this study. In our view, this illustrates the limitations of current approaches to treatment of CKD: therapy may prevent renal function deterioration but it is unable to promote regression on injury.
Since a major part of kidney mass was removed in this model, a persistent deterioration of renal function is expected with any therapy, which just prevents further deterioration of renal function. In this model, creatinine clearance is carried out with one-sixth of the total renal mass, equivalent to six times less nephrons than normal. No matter how effective the drug treatment is, physical limitation on the number of nephrons would not allow a serum creatinine decrease beyond a certain point. Lowering serum creatinine towards control values would require therapies that promote kidney regeneration.

Subtotal nephrectomy is a popular model to assess potential therapies for CKD and there is no consensus on the time point to start treatment. Some authors have started at Day 7 post-surgery [42]. However, previous studies had addressed the efficacy of late start (≥2 weeks after disease induction) of ARBs or statins in the subtotal nephrectomy model [7, 9, 43]. The difference in results between these studies and the present one may be related to the study design, as in our study, therapy was initiated earlier in the disease course than most previously reported experiences. This design is justified by the fact that recent awareness of the high prevalence of mild-to-moderate CKD as well as screening campaigns have resulted in a sharp increase in consultations to the nephrologist of these early-stage patients. Furthermore, certain human

![Graph showing renal endothelin-1 expression in 5/6 Nx rats.](https://academic.oup.com/ndt/article-abstract/27/7/2720/1844452/2728_A.Álvarez-Prats-et-al)
causes of renal mass reduction can be planned, such as unilateral nephrectomy plus removal of part of the other kidney for bilateral kidney tumors or may be diagnosed early as bilateral renal embolization, offering the opportunity for early intervention. In previous reports on the subtotal nephrectomy model, ARBs decreased but not
normalized proteinuria and decreased SBP and glomerulosclerosis, but did not preserve glomerular filtration rate [7, 9]. Our results differ in that renal function was better preserved in losartan-treated rats. The dose of losartan used in the present study only partially reduced albuminuria and renal injury in a longer term later start of treatment 5/6 Nx model. A previous report explored the effects of a statin (lovastatin) and/or an ACEI in a different model of subtotal nephrectomy where initiation of therapy was delayed beyond the length of our study [43]. Thus, the experimental model was not the same. Surprisingly, lovastatin decreased glomerulosclerosis but did not significantly reduce blood pressure or proteinuria or preserve renal function as assessed by blood urea nitrogen (BUN). Contrary to this report, but consistent with the lack of effect on proteinuria, we failed to observe a beneficial effect of statin therapy on glomerular injury. Again, contrary to this report, the statin preserved kidney function. This occurred despite a mild anti-hypertensive effect of the statin shared by both studies. As was the case for the ACEI-statin combination [43], the statin-ARB combination did not significantly add a benefit in most parameters of kidney injury. Only mesangial expansion was more effectively prevented by the ACEI-statin combination [43]. The modest anti-hypertensive property of statins has been previously reported [44, 45] and could be related to modulation of the synthesis of vasoactive mediators such as endothelin-1 and nitric oxide [45, 46].

NF-kB is a key transcription factor in kidney injury and therapeutic targeting of NF-kB is renoprotective [11, 15, 47]. The precise triggers for NF-kB activation in this model cannot be pinpointed from our studies. One possibility is that albuminuria triggers NF-kB activation. In this regard, angiotensin II and albumin promote renal cell NF-kB activation in culture and in vivo [48, 49]. This view would be supported by the correlation between albuminuria and NF-kB activation found in treatment-naive rats. Losartan had a striking anti-proteinuric effect but only a mild effect on renal NF-kB, suggesting the involvement of additional factors in remnant kidney NF-kB activation. The ARB–statin combination normalized NF-kB activity in the remnant kidney, while cerivastatin alone did not modulate NF-kB activity. A multi-drug approach which included RAS blockers and statin reduced renal injury in severe passive Heymann nephritis more than RAS blockade alone [50]. In this model, combination therapy further reduced kidney expression of inflammatory mediators as opposed to the modest effects of each drug alone [51].

Our results, although obtained in a different model, suggest that modulation of the activity of NF-kB, a key mediator of the inflammatory processes, may have contributed to these effects. Furthermore, in our model, activation of NF-kB closely correlated with glomerular and tubulointerstitial damage scores in treatment-naive animals. However, combined treatment did not provide additional clinical benefits despite complete normalization of NF-kB p65 activity. There are several potential explanations for this finding. One is that only a certain degree of NF-kB inhibition is required for maximal clinical benefit. In this regard, additional clinical benefit may be related to alternative biological mechanisms. Thus, a certain degree of NF-kB inhibition may prevent further injury, but additional clinical benefit may require, in addition, kidney regeneration and kidney regeneration might be NF-kB-independent. Another hypothesis is that additional molecular mediators contribute independently to the pathophysiological mechanisms of the disease. In this sense, co-activation of NF-kB and AP-1, rather than the simple activation of NF-kB, is related to the presence of kidney damage in patients with nephrotic syndrome [13]. On the other hand, the selective and efficient reduction of NF-kB in various experimental models does not guarantee the complete reversal of progressive renal disease [52, 53].

Glomerular and interstitial fibrosis is a key feature of CKD and type I and III collagens and fibronectin are main molecules of fibrotic extracellular matrix. The ARB-statin combination normalized the gene expression of fibronectin and interstitial collagen in the remnant kidney. In contrast, losartan or cerivastatin alone tended

![Fig. 9. Hypothetical integration of the study results. Based on current pathogenic concepts and the study results, we propose the following pathogenic cascade. We speculate that as a result of ARBs protection from glomerular injury, all subsequent events are downregulated. Thus, albuminuria, a known activator of NF-kB and inducer of tubulointerstitial injury, is decreased, rats are protected from tubulointerstitial injury and renal function is preserved. We acknowledge that physiopathology is usually complex and ARB may have beneficial effects at different points of this cascade, but we propose this as a working scheme. It is more difficult to explain the protective effect of cerivastatin on tubulointerstitial injury and renal function since it does not modulate glomerular injury, albuminuria or NF-kB activation. Thus, we have to propose either an effect downstream of NF-kB or on a parallel pathway, of which endothelin-1 may be a member or marker. This parallel pathway might have a mild effect on NF-kB activation, thus explaining the significant downregulation of NF-kB observed only in the presence of both drugs. In this regard, the combination of both drugs had a significant downregulatory effect on the expression of genes encoding extracellular matrix components. However, improved biochemical or histological injury parameters over losartan alone could not be demonstrated.](https://academic.oup.com/ndt/article-abstract/27/7/2720/1844452/Downloaded-from-https://academic.oup.com/ndt/article-abstract/27/7/2720/1844452?bibcode=201902March2019)
Combination therapy with an angiotensin II receptor blocker and a statin to decrease fibronectin and interstitial collagen expression. These results are consistent with the protective effect of all treatments over histological renal interstitial injury.

Statins may be renoprotective and we hypothesized that they might magnify the renoprotective effects of ARBs through their known pleiotropic properties, including the ability to attenuate the biological function of angiotensin II [54, 55], inhibiting ACE activity, thus contributing to achieving a full RAS blockade [50, 51, 56, 57]. Indeed, only an extremely high losartan dose, 10-fold higher than doses employed in this study, achieved complete AT1 blockade after renal ablation [30]. Cerivastatin blunts the increase in angiotensin II observed in dTGR rats [55]. Simvastatin prevents the adverse effects associated with chronic infusion of angiotensin II, such as the development of hypertension and the induction of reactive oxygen species [56]. Statins downregulate AT1 receptor expression through a Rho A-dependent mechanism [54]. In line with these findings, Rho-kinase has been linked to kidney damage caused by angiotensin II [58] and the inhibition of this pathway contributes to reducing harm to the kidney, due in part to the attenuation of the activity of NF-kB p65 [59]. Furthermore, similar to ARBs, statins attenuate NF-kB activity in diverse experimental systems, including in human mesangial cells [60]. However, the effect of statins on kidney NF-kB activity in the remnant kidney model had not been previously studied. In our study, cerivastatin alone did not prevent activation of NF-kB in nephrectomized rats. However, in the context of an attenuated renal disease as observed in animals treated with losartan, cerivastatin contributed to normalization of NF-kB activity, suggesting adjuvant properties for this statin in the reduction of renal disease progression. However, our results showing preservation of renal function and improvement of interstitial histology despite high NF-kB activity in rats treated with cerivastatin alone suggest that in this model modulation of NF-kB activity is not a key feature of nephroprotection offered by cerivastatin. Thus, nephroprotection by cerivastatin does not appear to share all of the molecular mechanisms with losartan. In this regard, losartan alone also decreased proteinuria, but cerivastatin did not.

If the beneficial effect of cerivastatin on serum creatinine and tubulointerstitial histology is not related to decreased albuminuria or inhibition of NF-kB activity, additional mechanisms must be postulated (Figure 9). In this regard, cerivastatin alone decreased total kidney preproendothelin-1 mRNA and endothelin-1 staining. Endothelin-1 has been implicated in kidney injury. Thus, endothelin targeting with bosentan partially prevented increases in blood pressure and proteinuria, but had a remarkable protective effect on renal function in the remnant kidney model [42]. In this regard, cerivastatin was associated with decreased plasma and urinary endothelin-1 levels in microalbuminuric patients with type 2 diabetic mellitus [21]. The fact that all treatments normalized pre-pro-ET1 gene expression, but not the expression of other mediators or NF-kB p65, suggests that molecular mediators other than ET-1 may independently contribute to the pathophysiological mechanisms of the disease. We thus hypothesize that ET-1 is a common target for both statins and ARBs that is situated early in the pathophysiological process, but whose normalization is unable to improve downstream events that may be activated by additional mediators.

In conclusion, ARB-statin combination therapy conferred certain advantages at the molecular level on kidney protection over ARB alone that are not clinically relevant after 6 weeks of therapy. Early therapy with ARB alone was anti-proteinuric, controlled SBP, and preserved renal function and glomerular and interstitial histology. The early use of statin alone, despite having no anti-proteinuric effect and only a mild effect on SBP, offered a similar benefit over serum creatinine and interstitial injury than ARB alone, suggesting different mechanisms of nephroprotection.

Supplementary data

Supplementary figure 1 is available online at http://ndt.oxfordjournals.org/.

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Lack of expression and function of erythropoietin receptors

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Abstract

Background. Erythropoiesis-stimulating agents (ESAs) stimulate formation of red blood cells by binding to and activating Epo receptors (EpoR) on erythroid progenitor cells. Beyond successful treatment of anemia, ESAs have been reported to reduce damage following insult to organs, including the kidney, possibly via direct activation of EpoR. However, data on ESA effects outside hematopoietic functions are conflicting. Furthermore, limited use of appropriate EpoR-positive and EpoR-negative controls and lack of specific anti-EpoR antibodies make interpretation of data difficult. Recently positive and negative control cell types were validated and a sensitive and specific anti-EpoR antibody (A82) that detects low levels of EpoR protein was described.

Methods. A82 was used to measure EpoR protein levels in tissues, human renal cells and human cell lines by western blot analysis. Surface EpoR was examined on renal cells by measuring binding of [¹²⁵I]-rHuEpo or antibodies. Renal cells and cell lines were treated with rHuEpo to see if phosphorylation of signaling proteins or proliferation/survival could be induced. Small inhibitory RNA (siRNA) were used to determine if EpoR knockdown affected cell viability.

Results. Total EpoR protein was low to undetectable in tissues and renal cells with no detectable EpoR on cell surfaces. EpoR knockdown had no effect on viability of renal cell lines. rHuhEpo had no detectable effect on intracellular signaling on renal cell lines with no growth-promoting or survival effect on primary human renal cells.

Conclusions. These results suggest that functional EpoR protein is absent on renal cells and that non-EpoR mechanisms should be explored to explain non-hematopoietic effects of ESAs.

Keywords: erythropoiesis-stimulating agents; erythropoietin; erythropoietin receptor; kidney; tissue protection

Introduction

Reports of improvement in organ function (including kidney) in animals with erythropoiesis-stimulating agent (ESA) treatment following various interventions [1, 2] suggested that ESAs may have broad effects. This formed the basis for clinical studies to examine a ‘tissue-protective’ effect of ESAs and potentially offered a mechanistic explanation for some observations in certain clinical studies with ESAs [2, 3].

The hypothesis that ESAs have direct effects on non-erythroid cells was spawned by reports that renal and


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