Antifibrotic, nephroprotective potential of ACE inhibitor vs AT1 antagonist in a murine model of renal fibrosis

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Abstract

Background. Several studies have shown antifibrotic effects of angiotensin converting enzyme (ACE) inhibitors as well as of angiotensin receptor 1 (AT1) antagonists, however, prospective trials with clinical end points comparing these effects do not exist. COL4A3−/− mice develop a non-hypertensive progressive renal fibrosis. We used this animal model to compare the potential of ACE inhibitor vs AT1 antagonist to prevent renal fibrosis irrespective of blood pressure-dependent involvement by the renin system.

Methods. COL4A3−/− mice were treated with placebo, ramipril or candesartan. Blood pressure, proteinuria, serum urea and lifespan were monitored. Renal matrix was characterized by immuno-histochemistry, light and electron microscopy. Further biochemical analysis was provided using cDNA microarray and western blot techniques.

Results. Untreated mice died of renal failure after 71 ± 6 days. Ramipril and candesartan both delayed onset and reduced the extent of proteinuria. Both had minor effects on blood pressure and postponed onset of uraemia. Ramipril increased lifespan by 111% to 150 ± 21 days (P < 0.01), whereas candesartan resulted in only a 38% prolongation to 98 ± 16 days (P < 0.01). Ramipril reduced glomerular and tubulo-interstitial fibrosis and numbers of activated fibroblasts to a greater extent than candesartan. Microarray and western blot analysis revealed a higher antifibrotic potential of ramipril in terms of downregulation of TGFβ, connective tissue growth factor, metalloproteinases and extracellular matrix proteins.

Conclusions. The results indicate an antifibrotic, nephroprotective effect of ACE inhibitors and AT1 antagonists in an animal model of progressive renal fibrosis. The greater antifibrotic effect of ramipril at the maximal therapeutic doses employed may not be explained by different antiproteinuric or blood pressure lowering properties, but by—in contrast to candesartan—its ability to hinder the proinflammatory, profibrotic activation of the angiotensin receptor 2.

Keywords: angiotensin; collagen; fibrinogen; renal hypertension; renal hypertension

Introduction

Both angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor 1 (AT1) antagonists have been shown to exhibit a nephroprotective potential in humans with chronic renal disease [1–3]. Partly, this can be explained by reduction of systemic blood pressure and proteinuria. However, few trials in humans compare these effects of ACE inhibitors vs those of AT1 antagonists. Remuzzi et al. found a similar potential of both drugs in terms of affecting proteinuria, microalbuminuria and albumin excretion [4]. Others report a trend towards a better effect of the ACE inhibitor [5]. Further mechanisms that protect renal function, such as the antifibrotic potential of these drugs, are less apparent. Moreover, it is still not clear whether ACE inhibitors or AT1 antagonists are superior in their ability to delay progressive fibrosis and renal failure [6,7].

The present study compares the antifibrotic potential of ACE inhibitor vs AT1 antagonist in COL4A3−/− mice [8]. These mice serve as an animal model for progressive renal fibrosis. Their phenotype is similar to the human Alport syndrome (AS) [8,9]. AS is a
hereditary nephropathy characterized by progressive renal failure, sensorineural deafness and typical ocular changes [10]. The disease in mice and men is caused by mutations in type IV collagen genes, leading to an abnormal composition of the glomerular basement membrane (GBM). Previous studies showed evidence that abnormal composition of the GBM leads to secondary events resulting in renal fibrosis [9]. We postulated that the nephrroprotective effect of ACE inhibitors and AT1 antagonists may influence some of those secondary events and delay glomerular, periglomerular and tubulointerstitial fibrosis.

Materials and methods

Animals

Genotyping of COL4A3−/− mice (Jackson Laboratory, Bar Harbor, Maine, USA) was carried out by PCR as described before [8,9]. Treatment protocols for the mice were previously approved by local German authorities and supervised by veterinarians. Mice were bred on a 129/SvJ genetic background.

Experiments

Systolic arterial blood pressure was measured using a non-invasive pressure cuff system (LE5001; Panlab, Barcelona, Spain). Ten microlitres of urine were used for micro-electrophoresis on a gradient polyacrylamide gel, a semi-quantitative technique used to qualify and quantify proteinuria [9]. Gels were stained with Coomassie blue and analysed by densitometry. Serum urea levels were analysed on a Hitachi 917 Automatic Analyzer (Boehringer Mannheim).

Ramipril (Aventis GmbH, Bad Soden, Germany) and candesartan (AstraZeneca GmbH, Wedel, Germany) were added to drinking water and replaced twice a week. Both drugs are stable in water at room temperature for more than 72 h, according to the suppliers; both drugs were given at the maximal therapeutic and well below the toxic range [11,12]. The disease in mice and men is caused by mutations in type IV collagen genes, leading to abnormal composition of the GBM. Previous studies showed evidence that abnormal composition of the GBM leads to secondary events resulting in renal fibrosis [9]. We postulated that the nephrroprotective effect of ACE inhibitors and AT1 antagonists may influence some of those secondary events and delay glomerular, periglomerular and tubulointerstitial fibrosis.

Results

Ramilpril extends lifespan of COL4A3−/− mice by 111%, candesartan only by 38%

Lifespan was continuously documented over a 15 month period (Figure 1). No animals were lost due to infections or adverse effects of therapy. The lifespan of untreated COL4A3−/− mice was 71 ± 5.7 days. The life expectancy of mice in group II (ramipril therapy) more
than doubled to 150±21 days ($P < 0.01, 99\%\ CI: 64–92$ days). The lifespan of candesartan-treated mice (group III) increased by only 38% to 98±16 days ($P < 0.01$, $99\%\ CI: 15–38$ days). The life expectancy of ramipril-treated mice was significantly longer than that of candesartan-treated mice ($P < 0.01$).

Both medical therapies have a similar effect on systolic blood pressure and reduce proteinuria

Mean systolic blood pressure was moderately increased in untreated COL4A3−/− compared with healthy controls in week 10, however, differences were too small to be significant in Student’s $t$-test (Table 1). Both therapies slightly reduced blood pressure in weeks 10 and 12. No differences were found between ramipril- and candesartan-treated animals. Both therapies reduced the level of proteinuria from 5 g/day in untreated animals to $< 2$ g/day in week 7 and from $\sim 12$ to $\sim 3.5$ g/day in week 10 ($P < 0.05$; Figure 2). Reduction of proteinuria was more than 50% in both therapy groups with no significant differences between ramipril- and candesartan-treated animals.

Both therapies delay onset of uraemia, however, deterioration of renal function is significantly slower in the ramipril therapy group compared with candesartan.

In untreated mice, serum urea started to rise above 50 mg/dl in week 7 (Figure 3) and to 247±27 mg/dl by week 9.5, followed by death soon after. In treated mice, deviation of urea was delayed by 3 weeks. By week 12, urea in ramipril-treated mice rose to $78\pm 19$ mg/dl, in contrast, urea in candesartan-treated mice was significantly higher ($146\pm 23$; $P < 0.05$).

### Table 1. Systolic mean arterial blood pressure (mmHg) of COL4A3−/− mice with and without medical treatment

<table>
<thead>
<tr>
<th></th>
<th>Wild-type placebo</th>
<th>Homozygous placebo</th>
<th>Homozygous ramipril</th>
<th>Homozygous candesartan</th>
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<tbody>
<tr>
<td>9.5 weeks</td>
<td>109±12</td>
<td>122±21</td>
<td>107±3</td>
<td>110±14</td>
</tr>
<tr>
<td>12 weeks</td>
<td>112±16</td>
<td>Animals dead</td>
<td>116±19</td>
<td>114±18</td>
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<td></td>
<td>($n = 5$)</td>
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<td>($n = 4$)</td>
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Ramipril therapy reduces abnormal deposition of the renal extracellular matrix as well as numbers of activated fibroblasts to a higher extent than candesartan.

Electron microscopy of COL4A3−/− mice showed characteristic thickening and splitting of the GBM irrespective of the therapy regime (Figure 4b–d). Abnormal intracellular amounts of fibrillar collagens and a complete loss of the podocyte food processes in untreated animals could be observed (Figure 4f). These changes appeared to be improved by candesartan therapy (Figure 4h) and even more improved by ramipril therapy (Figure 4g) as intact foot processes and slit-membranes could still be found.

Light microscopy showed marked periglomerular and tubulointerstitial fibrosis in untreated mice by week 10 (Figure 4j) with a complete loss of glomerular function and nephrons. Fibrotic changes of the glomerulum as well as of the tubulointerstitium were far less severe in the ramipril therapy group (Figure 4k).

A preserved glomerular architecture could as well be noted in the candesartan therapy group (Figure 4l), however, expansion of the mesangium and tubulointerstitium due to fibrosis was more severe than in ramipril-treated animals.

Immuno-histochemistry confirmed these changes (Figure 5): wild-type mice showed scant staining for fibronectin (Figure 5a). This was markedly increased in the periglomerular matrix of untreated COL4A3−/−, a typical finding of severe glomerulosclerosis (Figure 5b). Staining was markedly reduced in ramipril-treated animals (Figure 5c), while animals treated with candesartan (Figure 5d) showed a more intense signal of the periglomerular matrix.

Healthy controls showed a thin tubular and glomerular basement membrane (EHS-laminin, Figure 5e). In contrast, increased matrix deposition was seen in untreated animals (Figure 5f), laminin staining being present intra- and periglomerular, as well as in the intertubular regions. Localized shrinkage of tubular lumen was also noted, indicating loss of function of...
different nephrons. Periglomerular and intertubular signal was markedly decreased in the ramipril therapy group with an almost normal staining in the intertubular space and a preserved tubular lumen, suggesting a preserved function of nephrons (Figure 5g). In contrast, signalling was more prominent and localized loss of nephrons was found in the candesartan group (Figure 5h), indicating that candesartan therapy did not prevent periglomerular and tubulo-interstitial fibrosis to the same degree as ramipril did.

Staining for activated fibroblasts (Figure 6) confirmed these results: compared with the wild-type control (Figure 6a) untreated COL4A3-/− showed a very intense periglomerular and tubulointerstitial signal for α-smooth muscle actin (Figure 6b). This signalling was markedly reduced by ramipril therapy (Figure 6c), while being only mildly reduced in the candesartan therapy group (Figure 6d) with a scant periglomerular and localized tubulointerstitial signal.

For statistical analysis, glomeruli of three different animals of each group were evaluated for glomerulosclerosis in week 10 by a blinded observer. Glomerulosclerosis was defined as loss of more than 50% of glomerular lumen due to extracellular matrix accumulation. Two out of 88 (2.3%) glomeruli of healthy controls showed sclerosis, whereas 81 out of 92 (88.0%) did in untreated COL4A3-/−. In contrast, only 15 out of 90 (16.7%) of glomeruli of ramipril-treated COL4A3-/− and 36 out of 85 (42.4%) of glomeruli of candesartan-treated animals showed sclerosis. Tubulointerstitial fibrosis was evaluated in a similar manner by grading 12 different kidney sections of three different animals per experimental group (total of 36 sections) into zero, 1+ and 2+ accumulation of extracellular matrix by a blinded observer. Healthy controls showed no accumulation of extracellular matrix in any section; untreated COL4A3-/− showed an average matrix score of 1.83. Ramipril-treated mice showed a matrix score of 0.69 and candesartan-treated mice showed a matrix score of 0.93.

In cDNA microarray studies candesartan shows a stronger upregulation of profibrotic substances than ramipril

Compared with ramipril-treated animals, candesartan-treated mice showed a more than 2-fold stronger upregulation of TGFβ, MMP2, MMP9, laminin 1-subunits, nidogen, type I collagen and fibronectin (bold rows, Table 2). Furthermore, upregulation of other components such as type III collagen, α1-, α2-, α5-chain of type IV collagen, the type IV collagen receptors discoidin receptor 1 and integrin α2, and tissue inhibitor metalloproteinases I and II was demonstrated in the candesartan group when compared with the ramipril group.
Ramipril reduces TGFβ1 and CTGF protein expression to a higher extent than candesartan

We speculated that the nephroprotective effect of ramipril and candesartan in the mice may be due to downregulation of the profibrotic cytokines TGFβ1 and CTGF (Figure 7). Compared with untreated mice in weeks 7 and 10 (lanes 8 and 4), ramipril therapy (lanes 6 and 2) resulted in downregulation of TGFβ1 by more than 200% and CTGF by more than 400%. This effect was found to be weaker in the candesartan...
therapy group (lanes 7 and 3) (downregulation of TGFβ1 by only 80% and downregulation of CTGF by 100%).

Discussion

COL4A3−/− mice serve as a model of progressive renal disease leading to renal fibrosis and end-stage renal failure. All untreated animals showed a similar onset of uraemia and proteinuria resulting in death by 10 weeks of age. These reproducible changes allow observation of differences in the antifibrotic, nephroprotective potential of ACE inhibitors and AT1 antagonists. An antifibrotic effect of both drugs was clearly demonstrated: lifespan—the most evident end point—was prolonged by 111% in the ramipril therapy group and by 38% in the candesartan therapy group. Ramipril showed a significantly greater effect on lifespan than candesartan, but the reason for this is open to conjecture.

Both substances were given at the maximal therapeutic dosage in mice [11,12] and one would expect a maximal therapeutic effect of ramipril as well as of candesartan in our animal model. However, there were no tests performed to confirm equal inhibition of the renin system and therefore the present study did not fully characterize the dose–response relationship between the drugs tested and the changes observed in our animal model. Our present study showed a very similar effect of both drugs on blood pressure and on lowering proteinuria, indicating that both substances were similarly effective in lowering systemic and intraglomerular blood pressure. Furthermore, during the first 15 years of life, hypertension does not play a major part in the pathogenesis of renal fibrosis in AS [13]. Similarly, no significant differences in blood pressure were found in untreated vs treated mice (Table 1). Therefore, the antihypertensive effects of either drug do not explain the discrepancy in lifespan.

Urinary proteins have been shown to induce progressive interstitial fibrosis and the known antiproteinuric effects of ACE inhibitors and AT1 antagonists have been suggested to be nephroprotective. Our study demonstrates an antiproteinuric effect of both ramipril and candesartan. The similar antiproteinuric action of both drugs may well contribute to renoprotection in this model as it does in other models of progressive renal disease. However, it alone does not explain the significantly better lifespan in ramipril-treated mice.

Ramipril shows additional effects in preserving renal function in other human renal diseases [1]. ACE inhibitors block the conversion of angiotensin I to angiotensin II, a growth factor which activates fibroblasts leading to increased synthesis of matrix proteins [14]. Angiotensin II is also a profibrotic cytokine, activating mononuclear cells and increasing proinflammatory mediators [14], as well as regulating matrix degradation. Some of the downstream effects of angiotensin II are mediated via the angiotensin receptor 2 (AT2) [15] and directly via the TGFβ pathway which has been shown to be important in the renal fibrosis in AS [9,16]. Our data show that levels of TGFβ and CTGF are increased in untreated COL4A3−/− (Figure 7). Intervention with ramipril reduces TGFβ and CTGF expression more efficiently than candesartan does. This is paralleled by the deterioration in renal function. These results emphasize the role of TGFβ and CTGF in progression of renal fibrosis in the COL4A3−/−. Presumably, ramipril’s greater effect upon TGFβ and CTGF when compared with candesartan reduces glomerular, periglomerular and tubulointerstitial fibrosis more effectively.

Angiotensin II plays an important role in glomerular chemotaxis of macrophages and monocytes [15]. This induction and additional proinflammatory effects are mediated via the AT2 receptor [15,17,18]. In our animal model, kidneys of candesartan-treated mice showed more activated myofibroblasts (Figure 6) than kidneys of the ramipril group. Proinflammatory cells play a major role in developing tubulointerstitial fibrosis and loss of nephron function and have as well been shown to be key participants in the pathogenesis of renal fibrosis in AS [16]. AT1 antagonists do not block AT2 receptors, so the differences in interstitial fibrosis in ACE inhibitor- vs AT1 antagonist-treated animals suggests a role of AT2 receptors in developing renal fibrosis in our animal model.

Treatment of COL4A3−/− mice with ramipril showed a profound antifibrotic, nephroprotective effect, however, the effect on lifespan in Alport mice strongly depended on the time point when ramipril treatment was initiated. Ramipril might well be able to delay onset of renal failure and fibrosis in humans with AS [9,19]. We conclude that early therapy with an ACE inhibitor might be the first line treatment in children with AS. Therapy of AS with an AT1 antagonist seems to be less effective with regards to delay of renal failure and reduction of fibrosis and should be performed only if side effects of the ACE inhibitor, such as a cough, appear.

In summary, our study shows that ramipril and candesartan both reduce renal fibrosis and prolong lifespan in this animal model for progressive renal disease.
Therapy of progressive renal fibrosis

fibrosis. Both drugs influence profibrotic factors such as CTGF and TGFβ as important mediators of renal scarring. However, the prolongation of lifespan achieved by ramipril is much more pronounced than that in candesartan-treated animals, paralleled by stronger action against renal fibrosis. These nephroprotective data are in correspondence with the higher cardioprotective effect of the ACE inhibitor vs AT1 antagonist in the ELITE-II study [20]. The combination of both drugs may even have a stronger nephroprotective effect in our Alport mice than ramipril alone [19] and will be investigated further in the near future. Meanwhile, differences between ACE inhibitors and AT1 antagonists cannot be explained by their ability to reduce proteinuria or blood pressure. However, the stronger ’nephroprotective’ antifibrotic action of ramipril might be explained by its ability to block activation of the AT2 receptor via angiotensin II and therefore reduce its overt proinflammatory, profibrotic action.

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Conflict of interest statement. None declared.

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