Blockade of the Renin-Angiotensin and Endothelin Systems on Progressive Renal Injury

Zemin Cao, Mark E. Cooper, Leonard L. Wu, Alison J. Cox, Karin Jandeleit-Dahm, Darren J. Kelly, Richard E. Gilbert

Abstract—The renin-angiotensin system (RAS) and endothelin system may both play a role in the pathogenesis of progressive renal injury. The aims of the present study were 3-fold: first, to explore the possible benefits of dual blockade of the RAS with an ACE inhibitor and an angiotensin type 1 (AT1) receptor antagonist; second, to examine the relative efficacy of endothelin A receptor antagonism (ETA-RA) compared with combined endothelin A/B receptor antagonism (ETA/B-RA); and third, to assess whether interruption of both RAS and endothelin system had any advantages over single-system blockade. Subtotally nephrectomized rats were studied as a model of progressive renal injury and randomly assigned to one of the following treatments for 12 weeks: perindopril (ACE inhibitor), irbesartan (AT1 receptor antagonist), BMS193884 (ETA-RA), bosentan (ETA/B-RA), and a combination of irbesartan with either perindopril or BMS193884. Treatment with irbesartan or perindopril was associated with an improved glomerular filtration rate and reductions in blood pressure, urinary protein excretion, glomerulosclerosis, and tubular injury in association with reduced gene expression of transforming growth factor-β1 and matrix protein type IV collagen. The combination of irbesartan with perindopril was associated with further reductions in blood pressure and proteinuria. No beneficial effects of either BMS193884 or bosentan were noted. Furthermore, the addition of BMS193884 to irbesartan did not confer any additional benefits. These findings suggest that the RAS but not the endothelin system is a major mediator of progressive renal injury after renal mass reduction and that the combination of an AT1 receptor antagonist with an ACE inhibitor may have advantages over the single agent of RAS blocker treatment. (Hypertension. 2000;36:561-568.)

Key Words: kidney failure • angiotensin II • endothelin • transforming growth factors

Blockade of the renin-angiotensin system (RAS) with either an ACE inhibitor or angiotensin type I (AT1) receptor antagonist attenuates progressive renal injury in both experimental and clinical renal disease. Since the ACE inhibitor and AT1-receptor antagonist block the RAS at different sites, it might be expected that a combination of ACE inhibitor and AT1-receptor antagonist provide a greater degree of renoprotection than single-agent therapy.

Interaction between the RAS and endothelin (ET) pathways have been suggested to accelerate organ injury, with both vasoconstrictor systems increasing the expression of the prosclerotic cytokine transforming growth factor-β1 (TGF-β1). For instance, hypertension associated with infusion of ET is reduced by ACE inhibitors, and the vascular hypertrophy induced by angiotensin II (Ang II) infusion is not only associated with increased tissue ET but also can be reversed by an endothelin A receptor antagonist (ETA-RA).

The aims of the present study, conducted in a model of progressive renal injury, were, first, to explore the possible benefits of dual-agent compared with single-agent blockade of the RAS by use of an ACE inhibitor and/or an AT1 receptor antagonist; second, to examine the relative efficacy of ETA-RA compared with combined ETA/B receptor antagonist (ETA/B-RA); and third, to assess whether interruption of both the RAS and the ET system had any advantages over single-system blockade.

Methods

Experimental Protocol

Eight-week-old male Sprague-Dawley rats (body weight 220 to 310 g) housed at the Biological Research Laboratory at the Austin and Repatriation Medical Center were used. The protocols for animal experimentation and the handling of animals were in accordance with the principles set out by the Animal Welfare Committee of the Austin and Repatriation Medical Center. Subtotal nephrectomy (STNx; n=98) or sham surgery (control, n=16) was performed as described previously. In brief, the subtotal nephrectomy was performed by right nephrectomy, followed by infarction of approximately two thirds of the left kidney with selective ligation of all but one extrarenal branch of the left renal artery. Anesthesia was achieved by intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt, Boehringer Ingelheim). The subtotally nephrectomized animals were randomly allocated to one of the following drug therapies for 12 weeks: no drug therapy (STNx, n=16); an ACE
inhibitor, perindopril (Servier), at a dose of 8 mg/L in drinking water (STNx + perindopril, n = 16); an AT1-receptor antagonist, irbesartan (Bristol-Myers Squibb Pharmaceutical Research Institute), at a dose of 15 mg/kg per day by gavage (STNx + irbesartan, n = 14); an ETA-RA, BMS193884 (Bristol-Myers Squibb Pharmaceutical Research Institute), at a dose of 100 mg/kg per day by gavage (STNx + BMS193884, n = 11); a dual treatment, ETA-B/RA and bosentan (Hoffman-La Roche), at a dose of 100 mg/kg per day by gavage (STNx + bosentan, n = 15); and combination of irbesartan with either perindopril (STNx + irbesartan + perindopril, n = 14) or BMS193884 (STNx + irbesartan + BMS193884, n = 12). The doses of irbesartan and perindopril were chosen according to previous studies.5,11

The rats had unrestricted access to water and standard rat chow (Clark King & Co). Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Perindopril, bosentan, and BMS193884 were administered by gavage every 4 weeks after operation. At 24 hours for collection of urinary samples and measurement of renal function including glomerular filtration rate (GFR), plasma creatinine concentration and urinary protein excretion were determined. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized rats.

### Gene Expression of TGF-β1 and Type IV Collagen

Quantitative in situ hybridization that permits the quantification of gene expression was used to determine the magnitude of gene expression with established methods as previously reported.5,16–18 Antisense riboprobes for TGF-β1, and type IV collagen were generated as previously described.19,20 In brief, a 985-bp cDNA probe coding for rat TGF-β1, (gift of Dr Qian, NIH, Bethesda, Md) was cloned into pBluescript KS (+, Stratagene) and linearized with XhoI, and an antisense riboprobe was produced with T7 RNA polymerase. The 1.8-kb cDNA probe coding for mouse type IV collagen (gift of Dr R. Timpl, Max Planck Institute, Martinsried, Germany) was cloned into pGEM 3Z and linearized with BamHI to produce an antisense riboprobe with SP6 RNA polymerase. Sections were hybridized with riboprobes and then exposed to Kodak X-Omat autoradiographic film for 3 days. Film densitometry of autoradiographic images obtained by in situ hybridization was assessed with the use of a microcomputer imaging device (MCID, Imaging Research) with an associated video camera and an IBM AT computer as previously described.5,16

### 125I-Endothelin Binding

To determine if blockade of ETA receptors in the kidney occurred with bosentan or BMS193884, 125I-endothelin I binding was assessed in a separate group of animals by means of in vitro autoradiography as previously reported.21 In this experiment, rats were given bosentan (100 mg/kg), BMS193884 (100 mg/kg), or vehicle by gavage (n = 3 per group). The animals were anesthetized with intravenous injection of sodium pentobarbital (60 mg/kg body wt). A midline incision of the abdomen was made, and remnant kidneys were removed, weighed, and fixed in 10% formalin and embedded with paraffin. Four-micron paraffin sections of kidney were used for histopathology and in situ hybridization.

### Assessment of Renal Function

Renal function including glomerular filtration rate (GFR), plasma creatinine concentration and urinary protein excretion were determined at the conclusion of the experiment. GFR was measured by use of the 99mTc-DTPA method.15 Plasma urea and creatinine concentrations were measured with the use of an autoanalyzer (Beckman Instruments). Animals were housed in metabolic cages for 24 hours for collection of urinary samples and measurement of urinary protein excretion with the Coomasie brilliant blue method.14

### Kidney Histopathology

Assessment of glomerulosclerosis and tubulointerstitial injury was performed with the use of semiquantitative scores described previously.5,12 Kidney sections were stained with hematoxylin and eosin and observed under light microscope in a masked fashion at a magnification of ×400. Thirty glomeruli in each kidney were graded according to the severity of the glomerular damage: 0, normal; 1, slight glomerular damage, the mesangial matrix and/or hyalinosis with focal adhesion, involving <25% of the glomerulus; 2, sclerosis of 25% to 50%; 3, sclerosis of 50% to 75%; and 4, sclerosis of >75% of the glomerulus. Twenty fields of tubulointerstitial area in the cortex were observed and graded as follows: 0, normal; 1, the 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. Indexes of glomerular damage or tubulointerstitial lesion were calculated by averaging the grades assigned to all glomeruli or tubular fields.

### Table 1. Body Weight, Urinary Volume, and Kidney Weight

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Wk 0</th>
<th>Wk 12</th>
<th>Weight gain</th>
<th>Urinary Volume, mL/24 h</th>
<th>Kidney Weight, g</th>
<th>Kidney/Body Wt Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16</td>
<td>259±8</td>
<td>502±13</td>
<td>256±15</td>
<td>21±1</td>
<td>1.42±0.05</td>
<td>0.28±0.01</td>
</tr>
<tr>
<td>STNx</td>
<td>16</td>
<td>265±8</td>
<td>432±14</td>
<td>160±15*</td>
<td>41±4*</td>
<td>1.85±0.09*</td>
<td>0.43±0.02*</td>
</tr>
<tr>
<td>STNx + perindopril</td>
<td>16</td>
<td>298±5</td>
<td>471±14</td>
<td>173±15*</td>
<td>37±3*</td>
<td>1.97±0.11*</td>
<td>0.42±0.02*</td>
</tr>
<tr>
<td>STNx + irbesartan</td>
<td>14</td>
<td>225±4</td>
<td>410±7</td>
<td>185±8*</td>
<td>36±2*</td>
<td>1.14±0.03†</td>
<td>0.28±0.01†</td>
</tr>
<tr>
<td>STNx + BMS193884</td>
<td>11</td>
<td>214±4</td>
<td>402±14</td>
<td>188±18*</td>
<td>45±4*</td>
<td>2.08±0.13*</td>
<td>0.55±0.03†</td>
</tr>
<tr>
<td>STNx + bosentan</td>
<td>15</td>
<td>220±4</td>
<td>404±12</td>
<td>181±13*</td>
<td>42±4*</td>
<td>2.19±0.11*</td>
<td>0.53±0.04†</td>
</tr>
<tr>
<td>STNx + irbesartan + perindopril</td>
<td>14</td>
<td>297±5</td>
<td>349±6</td>
<td>93±7†</td>
<td>40±2*</td>
<td>1.25±0.05†</td>
<td>0.36±0.04†</td>
</tr>
<tr>
<td>STNx + irbesartan + BMS193884</td>
<td>12</td>
<td>233±4</td>
<td>408±12</td>
<td>175±11*</td>
<td>37±2*</td>
<td>1.64±0.07</td>
<td>0.40±0.02*</td>
</tr>
</tbody>
</table>

*P<0.05 vs control; † P<0.05 vs STNx.
nephrectomized rats compared with those in control rats. Only irbesartan alone or in combination with perindopril reduced these parameters (Table 1). Urinary volume was nearly doubled in subtotally nephrectomized rats than in control and was not influenced by any treatment (Table 1). Subtotally nephrectomized rats were associated with elevated SBP (Table 2 and Figure 1A). The rise in SBP after subtotal nephrectomy was ameliorated by interventions, which blocked the RAS but not the endothelin antagonists BMS193884 and bosentan when used as single-agent therapy (Table 2 and Figure 1A). The combination of irbesartan and perindopril was associated with a lower mean SBP than observed with either agent administration as monotherapy (Figure 1A). There was no significant difference in SBP reduction by the addition of BMS193884 to irbesartan compared with irbesartan therapy alone.

### Renal Function

Urinary protein excretion was significantly increased in renally ablated rats and was reduced by all treatments except bosentan and BMS193884 (Figure 1B). Both irbesartan and perindopril reduced proteinuria with the combination of these two agents, achieving an even greater reduction in proteinuria than either treatment alone. The combination of irbesartan with BMS193884 did not reduce proteinuria more than with irbesartan alone.

Plasma urea and creatinine concentrations were markedly increased in nephrectomized rats compared with control animals (Table 2). Treatment with perindopril or irbesartan alone or the combination of both resulted in serum urea and creatinine concentrations to a similar extent. Both bosentan and BMS193884 did not affect these parameters. Similarly, GFR was markedly reduced in subtotally nephrectomized rats and ameliorated by all therapies apart from bosentan and BMS193884 (Figure 1C).

### Table 2. SBP, Plasma Urea, and Creatinine Values

<table>
<thead>
<tr>
<th>Group</th>
<th>SBP, mm Hg</th>
<th>Plasma Urea, mmol/L</th>
<th>Plasma Creatinine, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wk 4</td>
<td>Wk 8</td>
<td>Wk 12</td>
</tr>
<tr>
<td>Control</td>
<td>122±2</td>
<td>127±4</td>
<td>130±2</td>
</tr>
<tr>
<td>STNx</td>
<td>159±7*</td>
<td>179±7*</td>
<td>188±5*</td>
</tr>
<tr>
<td>STNx + perindopril</td>
<td>116±3†</td>
<td>131±4†</td>
<td>141±6†</td>
</tr>
<tr>
<td>STNx + irbesartan</td>
<td>127±5†</td>
<td>125±5†</td>
<td>119±5†</td>
</tr>
<tr>
<td>STNx + BMS193884</td>
<td>163±7*</td>
<td>159±9*</td>
<td>185±7*</td>
</tr>
<tr>
<td>STNx + bosentan</td>
<td>154±3*</td>
<td>181±7*</td>
<td>177±5*</td>
</tr>
<tr>
<td>STNx + irbesartan + perindopril</td>
<td>106±2†</td>
<td>116±3†</td>
<td>110±4†</td>
</tr>
<tr>
<td>STNx + irbesartan + BMS193884</td>
<td>131±6†</td>
<td>118±3†</td>
<td>123±5†</td>
</tr>
</tbody>
</table>

*P<0.05 vs control; †P<0.05 vs STNx.
Kidney Histology
Glomerulosclerosis and tubulointerstitial injury occurred in untreated STNx rats and were reduced in all groups except those treated with ET antagonists alone (Figure 2). The combination of irbesartan with perindopril or BMS193884 did not reduce glomerulosclerosis or tubulointerstitial injury to a greater extent than when irbesartan or perindopril was used as single-agent therapy.

Gene Expression of TGF-β and Type IV Collagen
Overexpression of both TGF-β and type IV collagen mRNA was observed in untreated STNx rats (Figures 3, 4, and 5). Expression of both transcripts was reduced in all groups except those treated with the endothelin antagonists, bosentan and BMS193884. Combination of irbesartan with perindopril or BMS193884 did not reduce TGF-β and type IV collagen mRNA to a greater extent than when irbesartan or perindopril was used as monotherapy.

125I-Endothelin Binding
The major binding sites of the endothelin I in the vehicle-treated kidney were detected in the cortex and medulla, as previously reported21 (Figure 6). Compared with the 125I-endothelin I binding in cortex (132±8 dpm/mm²) and medulla (125±15 dpm/mm²) in vehicle-treated kidney, administration of bosentan was able to reduce 125I-endothelin I binding both in cortex (33±2 dpm/mm²) and medulla (23±4 dpm/mm², $P<0.05$ versus vehicle, respectively). Gavage of BMS193884 was also associated with a reduction of 125I-endothelin I binding in the cortex (70±9 dpm/mm²) and medulla (45±6 dpm/mm², $P<0.05$ versus vehicle, respectively).

Discussion
The present study demonstrates that in rats with reduced renal mass, the addition of AT1 receptor antagonist therapy to ACE
inhibitor treatment resulted in greater reductions in both blood pressure and proteinuria than single-agent treatment. By contrast, antagonism of either ET receptor was without effect either as sole or as additional therapy to blockade of the RAS.

ACE inhibitors reduce the rate of progression in both diabetic and nondiabetic renal disease. However, although progression is slowed, it is not arrested, indicating the need for adjunctive therapy. Indeed, in long-term studies, Ang II concentrations with chronic ACE inhibitor are not reduced.22 This is believed to be a consequence of the ACE inhibitor–induced rebound increase in renin activity that ultimately leads to enhanced Ang II formation. Similarly, with the AT1-receptor antagonist, the rebound increases in both renin and Ang II may overcome the effects of receptor blockade. Thus, blocking both the formation of Ang II with an ACE inhibitor and its receptor binding with an AT1-receptor antagonist may have additive effects beyond single-agent treatment.

The present study emphasized a predominant role of blood pressure reduction rather than the blockade of the RAS in conferring renoprotection in this model. Further support for this concept is provided from a study in which a combination of ACE inhibitor and AT1-receptor antagonist was used in doses to achieve blood pressure levels similar to monotherapy with either agent.7 These investigators showed that the combination did not confer superior renoprotection to single-agent treatment. Menard et al23 have explored the role of combination treatment with losartan and enalapril on blood pressure and cardiac hypertrophy in spontaneously hypertensive rats. This combination was effective at reducing cardiac hypertrophy but in the context of superior blood pressure reduction.23 Nevertheless, one cannot exclude a potential additive role for blockade of the RAS in mediating organ protection from hypertension-induced injury. For example, a recent study exploring the use of perindopril and candesartan in stroke-prone spontaneously hypertensive rats suggested a role for this combination in reducing left ventricular weight.24 This effect appeared to be more than one would have predicted from reduction in blood pressure alone.24 The importance of the specific action of antihypertensive drugs has been further explored by the pivotal studies of Griffin et al,2,25 who have compared ACE inhibition to various calcium channel blockers in the subtotal nephrectomy model. Their studies, which have included accurate radiotelemetric assessment of blood pressure, confirmed that ACE inhibition reduced proteinuria in association with blood pressure reduction, yet calcium channel blockers despite reducing blood pressure failed to improve renal function and pathology in these renally ablated rats.2,25

Although the combination of perindopril and irbesartan reduced blood pressure and proteinuria, it did not lessen the reduction in GFR compared with single-agent therapy. However, the difference in GFR between groups may not be a sensitive marker of response to treatment.1,26 For instance, in the REIN study, the decline in GFR per month in patients with nondiabetic renal disease and 1 to 3 g/d of proteinuria was not significantly different in ramipril-treated patients, although progression to end-stage renal failure was significantly less common in the ACE inhibitor–treated group.26 These findings are consistent with the view that proteinuria may be used as a marker for progression of renal injury27,28 and that in a therapeutic setting, renoprotective therapy should be titrated against urinary protein excretion as well as blood pressure.29 Thus, the findings of the present study suggest the potential for the combination of ACE inhibitor with AT1-receptor antagonist as a therapeutic strategy in patients with progressive renal disease30 in addition to its role in the treatment of hypertension31 and cardiac failure.32

TGF-β has been consistently implicated as playing a pivotal role in the pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis in progressive renal disease of diverse pathogeneses.33 Indeed, in vitro studies suggest that the fibrogenic effects of Ang II are mediated by TGF-β34 and that in vivo there is a dose-response relation between TGF-β production and both enalapril and losartan treatment.35 However, if both drugs were added in their maximal effective doses, no additional effect on TGF-β production was observed.35 In the present study, the combination of perindopril with irbesartan did not further diminish TGF-β expression or structural injury compared with single-agent therapy, although blood pressure and proteinuria were both further improved. These findings suggest that the mechanisms underlying the dose-response relation between Ang II and TGF-β may be different from those of Ang II and blood pressure and Ang II and proteinuria.

In contrast to ACE inhibition and AT1-receptor antagonist, the present study found that endothelin antagonism was ineffective in lowering blood pressure, decreasing proteinuria, or slowing the decline in GFR in subtotally nephrectomized rats. The lack of renoprotective effects of bosentan and BMS193884 in the present study are not likely to be due to inadequate blockade of ET receptors, as in vitro autoradiog-
raphy demonstrated blockade of ET receptors by bosentan and BMS193884 administered in the doses used in the present study. The findings in the present study are consistent with several but not all of the experiments that have examined the effects of ET antagonism in this model. For instance, Potter and colleagues reported that despite abrogating the blood pressure rise associated with renal mass reduction, treatment with the ETA-RA PD155080 did not alter urinary protein excretion or creatinine clearance. In other studies, not even the blood pressure rise was ameliorated with either dual

Figure 6. Representative macroscopic autoradiographs of $^{125}$I-endothelin I binding in the kidney 4 hours after gavage with vehicle (A), bosentan (B), and BMS193884 (C).
ETA/B (Ro 46-2005) or ETA-receptor antagonism (A-127722 and BMS182874). In contrast to these previous studies and the present study, another group has reported that both bosentan and the ETA-RA FR 139317 reduced blood pressure, proteinuria, and declining GFR in rats with renal mass reduction. It remains to be determined as to why there were differences in the effects of ET antagonism on blood pressure and renal function in the various studies. There were differences in protocols, periods of treatment, and the types of ET antagonist used in the various studies, and these factors may partly explain the disparity in results.

A close interrelation between ET-I and Ang II in relation to their effects on mesangial cell proliferation and extracellular matrix synthesis has been described, leading us to hypothesize that further benefits on blood pressure and renal function may be possible when ET-receptor antagonist is added to RAS blockade. However, the combination of ET antagonist and AT1-receptor antagonist was not associated with further renal protection than observed with AT1-receptor antagonist alone, consistent with the view that the major approach for reducing injury in this model is by blockade of Ang II–dependent rather than ET-dependent pathways.

Administration of irbesartan was associated with reduced kidney weight when compared with perindopril-treated animals. Similarly, rats treated with the combination of irbesartan and perindopril had reduced kidney weight when compared with those treated with perindopril. Furthermore, in the irbesartan-plus-perindopril–treated group, there was also reduced weight gain. A similar effect of the combination of ACE inhibitor and AT1-receptor antagonist on body weight has also been observed by another group. The mechanisms underlying these differences in both kidney and body weight are uncertain. It is unlikely that the renal benefits of irbesartan in the present study were due to reduced kidney weight, since irbesartan treatment was associated with GFR, serum urea, and creatinine levels similar to those observed in rats treated with perindopril. Since irbesartan- or perindopril-treated rats gained similar weight over the period of experiment, a lower kidney weight and a subsequent lower kidney/body weight ratio in irbesartan treatment either as monotherapy or in combination may represent a specific antitrophic effect of this agent, which warrants further examination.

In summary, after renal mass reduction, the combination of ACE inhibitor with AT1-receptor antagonist led to further reductions in blood pressure and proteinuria than did single-agent therapy, suggesting the potential for this approach in the treatment of renal disease in humans, where therapeutic targets include optimization of blood pressure and reduction in proteinuria.

Acknowledgments

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