Mechanisms underlying renoprotection during renin-angiotensin system blockade

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Early treatment of rats subjected to extensive renal mass ablation with angiotensin-converting enzyme inhibitors (ACEI) effectively prevents the focal and segmental glomerulosclerosis (FSGS) and tubulointerstitial fibrosis (TIF) that ensues in untreated rats, an effect attributable, in part, to normalization of the raised glomerular capillary hydraulic pressure (Pgc) characteristic of this model of chronic renal disease (CRD) progression (3, 4). Because these experimental findings were later matched in landmark clinical trials (14, 27, 30, 40, 45, 46), ACEI therapy is now widely regarded as a fundamental component of strategies designed to retard the progression of CRD. Angiotensin subtype 1 receptor antagonists (AT1RA), a novel class of antihypertensives, inhibit the renin-angiotensin system (RAS) distal to angiotensin-converting enzyme (ACE) and may offer therapeutic advantages over ACEI (51). Nevertheless, previous studies from this and other laboratories detected no significant differences in the renoprotective effects of ACEI vs. AT1RA in experimental models of CRD, when treatment was initiated before the onset of substantial renal injury (2, 5, 19, 21, 26, 36, 41, 47, 58, 60). A single study, however, purports to show an advantage of AT1RA over ACEI in 5/6 nephrectomized rats (34). Notably, in this study treatment was initiated only after renal injury was evident and was of considerably longer duration than previous studies. These findings raised the possibility that subtle benefits of AT1RA over ACEI may become evident only over an extended time period in a model in which RAS blockade results in a slowing but not an arresting of CRD progression. This is particularly important because clinical trials of ACEI treatment in CRD have also observed a slowing rather than an arresting of CRD progression (14, 27, 30).

Because the therapeutic ideal is to arrest or even reverse CRD progression, it is important to identify factors that may contribute to the slow progression of CRD during RAS inhibition. Systemic blood pressure has been shown in experimental (6) and clinical (20, 22, 23, 32, 37, 39) studies to be an important determinant of chronic renal injury, but the role of blood pressure in the context of ACEI treatment requires further elucidation. Proteinuria, long regarded as a marker of glomerular injury, has recently been proposed as an important factor contributing to the pathogenesis of CRD progression (42). Finally, recent studies have found

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that extensive renal mass ablation provokes the coordinated induction of several proinflammatory genes and infiltration of the remnant kidney by macrophages (48, 53, 57). These chronic inflammatory responses are prevented by early treatment with ACEI or AT1 RA, suggesting that macrophages and a variety of proinflammatory molecules may contribute to the pathogenesis of progressive renal fibrosis (53, 57). We hypothesized that persistent upregulation of inflammatory and profibrotic gene expression may also be a significant factor associated with slow progression of renal injury during ACEI or AT1 RA therapy.

In this study we utilized relatively large numbers of rats in a delayed treatment protocol with prolonged follow up to 1) determine whether, at doses that produce equivalent antihypertensive effects, the AT1 RA, candesartan, and the ACEI, enalapril, share equivalent renoprotective effects; and 2) examine the role of systemic blood pressure and proteinuria as well as renal inflammatory and profibrotic gene expression in contributing to the slow progression of CRD during RAS inhibition.

METHODS

Animals. Adult male Munich-Wistar rats (218–278 g) were obtained from Simonsen Laboratories (Gilroy, CA), housed under standard conditions, and given unrestricted access to standard rodent chow and water. Rats were subjected to either renal mass ablation by right nephrectomy and ligation of two or three branches of the left renal artery, producing infarction of approximately two-thirds of the left kidney (n = 63), or sham operation by laparotomy and mobilization of the renal vessels (Sham rats; n = 15). All surgical procedures were performed under pentobarbital anesthesia (Nembutal, 50 mg/kg ip; Abbott Laboratories, Chicago, IL). At 2-wk intervals, systolic blood pressure (SBP) was measured by the tail-cuff method, and daily urinary protein excretion rate (Upro) was determined on urine collected from rats individually housed in metabolic cages for 24 h. At 5 wk after renal mass ablation, rats were stratified according to SBP and Upro and allocated to the following groups. Csn rats (n = 30) received candesartan cilexetil (TCV-116; Takeda Chemical Industries, Osaka, Japan; 50 mg/l (3–7 mg·kg−1·day−1) in the drinking water). Vehicle comprising ethanol (0.1%, vol/vol), polyethylene glycol (0.1%, vol/vol), and sodium bicarbonate (5 mmol/l) was added to achieve water solubility of candesartan. Ena rats (n = 27) received enalapril [Merck Research Laboratories, Rahway, NJ; 110 mg/l (7–15 mg·kg−1·day−1) in the drinking water with sodium bicarbonate (5 mmol/l)]. Some dosage adjustments were made initially to achieve equivalent blood pressure control in the treatment groups. Six rats were killed at 5 wk after renal ablation, and the remnant kidneys were harvested to provide pretreatment data (5WK rats; n = 6). The remaining rats were studied for a total of 12 (set A: CsnA, n = 11; EnaA, n = 11; ShamA, n = 6) or 24 wk (set B: CsnB, n = 19; EnaB, n = 16; ShamB, n = 9). At the end of the observation period, rats were anesthetized with pentobarbital, and portions of renal cortex distant from the infarct scar were excised and snap frozen in liquid nitrogen for subsequent RNA extraction and immunohistology. The remnant kidney was then perfusion-fixed with 1.25% glutaraldehyde in 0.1 mmol/l sodium cacodylate buffer (pH 7.4), delivered through a catheter in the abdominal aorta at the measured SBP of each rat. Kidneys were weighed after perfusion fixation. To evaluate renal hypertrophy, final remnant kidney weight, corrected for the increase in weight associated with perfusion fixation, was compared with an estimate of baseline remnant kidney weight taken as one-third of the weight of the right kidney removed at the time of renal mass ablation. The correction factor (1.38) was derived by comparing the weights of the unfixed right kidney with those of the perfusion-fixed left kidneys removed contemporaneously from 15 sham-operated rats.

Untreated controls were not included in the study because, on the basis of previous experience in our laboratory, the chance for survival to 24 wk was expected to be close to zero. For purposes of comparison, data from untreated control rats after 5/6 nephrectomy were pooled from previous 12-wk studies recently performed in this laboratory. The percentage of glomeruli affected by sclerosis in 22 untreated rats at 12 wk after 5/6 nephrectomy was 43 ± 17 (SD) % (29, 36).

Morphology. Renal tissue was postfixed in 10% phosphate-buffered Formalin, embedded in paraffin, and processed for light microscopy. The frequency of FSGS was estimated by examining all glomeruli seen in one or two coronal sections from each kidney stained by the periodic acid-Schiff method. Segmental sclerosis was defined as glomerular capillary collapse with hyaline deposition and/or adhesion to the parietal layer of Bowman’s capsule. A glomerulosclerosis score (GS) was determined by expressing the number of glomeruli with segmental or global sclerosis as a percentage of the total number of glomeruli counted for each rat (mean 132; range 73–274/rat). Tubulointerstitial injury, as evidenced by dilated tubules containing protein casts and interstitial inflammation or fibrosis, was assessed at medium power on the same sections before evaluation of the glomerulosclerosis. A scoring system (tubulointerstitial score (TIS)) was used to grade the injury from 0 to 3 on the basis of the percentage of abnormal tissue (0, <20, 20–50, and >50%, respectively). The pathologist was unaware of the group assignment of individual rats.

Chemical analysis. The concentration of protein in the urine was determined by spectrophotometry after precipitation with 3% sulfosalicylic acid.

Competitive RT-PCR. Total RNA was extracted from frozen portions of renal cortex by the cesium chloride ultracentrifugation method (11). RNA was quantitated by determination of ultraviolet absorbance at 260 nm, and its purity was assessed by measuring the optical density ratio at 260 and 280 nm. For preparation of cDNAs, 4 μg of heat-denatured RNA were used in an RT reaction. The entire sample in a total volume of 20 μl contained 4 μg of RNA; 0.5 mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech, Piscataway, NJ); 0.5 μg oligo-d(T)12−18 (Pharmacia Biotech); 40 U RNasin ribonuclease inhibitor (Promega, Madison, WI); and 200 U Moloney murine leukemia virus RT (Life Technologies, Gaithersburg, MD) in a buffer of (in mM) 50 Tris·HCl (pH 8.3), 75 KCl, 3 MgCl2, and 10 dithiothreitol. The solution was incubated for 60 min at 37°C and then held at 95°C for 5 min to arrest the reaction.

Preparations of cDNA were then used as substrate for competitive PCR reaction by using competitive DNA mimics and oligonucleotide primer sets (Genosys Biotechnologies, Woodlands, TX). Competitive DNA mimics for each factor, comprising a segment of neutral DNA with sequences complimentary to the gene-specific primers attached to each end, were constructed by using a PCR MIMIC construction kit (Clontech Laboratories, Palo Alto, CA). Primer sets were designed for rat transforming growth factor (TGF)−β1, monocyte chemoattractant protein (MCP)−1, interleukin (IL)−1β,
starting reaction by using the formula described (21, 55) from the known amount of the mimic in the template (Fig. 1, A and B). Thus absolute amounts of RNA from unknown samples were calculated as previously described (21, 55) from the known amount of the mimic in the starting reaction by using the formula

\[
\text{[target gene]} = \frac{[\text{mimic}]}{([\text{target gene product}] / [\text{mimic product}])}^{\alpha}
\]

where \( \alpha \) is the gradient of the log plot of target gene product-mimic product vs. serial dilutions of starting cDNA (Fig. 1B). Specimens were run in duplicate, and the average value was used. We have previously established that this assay is readily capable of detecting a twofold difference in target gene concentration (21, 53). As the number of specimens exceeded the capacity of the thermal cycler, all specimens from the study could not be included in a single PCR reaction. Specimens from 5WK, CsnA, EnaA and Sham a rats were therefore included in one set of PCR reactions and specimens from 5WK, CsnB and Enab, in a separate set of PCR reactions. To allow direct comparison of results from different PCR reactions, data were expressed as ratios to the mean value for the 5WK group. \( \beta \)-Actin mRNA levels were used to confirm that starting amounts of cDNA were similar among groups.

**Immunohistochemistry.** Expression of TGF-\( \beta \), MCP-1, and IL-1\( \beta \) proteins and macrophage infiltration was assessed by immunohistochemistry. For macrophage staining, 4-\( \mu \)m paraffin sections of fixed tissues were used for immunoperoxidase analysis after baking at 60°C for 1 h, deparaffinization, and rehydration (xylene \( \times 4 \) for 3 min each, 100% ethanol \( \times 4 \) for 3 min each, and running water for 5 min). The sections were then microwave treated at 93°C for 30 min in preheated 10 mM citrate buffer, pH 6.0, cooled for 15 min, and transferred to PBS. Sections were then blocked (for 15 min) with a 1.5% solution of serum from the animal source of the secondary antibody at room temperature. Next, sections were incubated with primary antibody (dilution 1:1000) at 4°C for 18 h. After washing in PBS, sections were incubated with the secondary antibody at room temperature. Next, they were washed in PBS, incubated for 20 min in 0.05% diaminobenzidine, and counterstained with hematoxylin. Sections were then dehydrated, cleared, and mounted.

**Table 1. Summary of primer sequences and PCR conditions**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense Sequence</th>
<th>Antisense Sequence</th>
<th>Size, bp</th>
<th>Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-( \beta )</td>
<td>CTTGAGCTCCAGAGAGAAACTGCG</td>
<td>CAGACATGTCATTGGAAGAATGCTGC</td>
<td>298</td>
<td>64</td>
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<tr>
<td>MCP-1</td>
<td>ATGCAAGTCGTCGTGACAG</td>
<td>CTGTTTCCTGTGATCATCTT</td>
<td>447</td>
<td>55</td>
</tr>
<tr>
<td>IL-1( \beta )</td>
<td>TGGGTTCGTGTCATTAGACAGC</td>
<td>GAGGTGCTGATGTACCAGTT</td>
<td>378</td>
<td>55</td>
</tr>
<tr>
<td>( \beta )-Actin</td>
<td>TGGTAAAGGACACCTGCGAGGATATGG</td>
<td>GATCTGCTCCTGCTGTGGTAG</td>
<td>764</td>
<td>60</td>
</tr>
</tbody>
</table>

TGF-\( \beta \), transforming growth factor-\( \beta \); MCP-1, monocyte chemoattractant protein-1; IL-1\( \beta \), interleukin-1\( \beta \). *Annealing temperature.
MECHANISMS OF RENOPROTECTION DURING RAS BLOCKADE

were incubated with mouse monoclonal antibodies to the monocyte/macrophage marker ED1 (clone ED1, 1:150 dilution, BioSource International, Camarillo, CA) for 1 h in a humid chamber at room temperature. The secondary antibody (Vectastain Elite ABC Kit, Vector Laboratories, Burling- ton, CA) was used according to manufacturer’s instructions. Slides were rinsed with PBS between each incubation. Sections were developed by using 3,3'-diaminobenzidine (Sigma, St. Louis, MO) as substrate and counterstained with Gill’s hematoxylin (Fisher Scientific, Pittsburgh, PA). Macrophage infiltration was assessed by counting the number of ED1-positive cells in 10 glomerular profiles and in 10 randomly chosen 0.25 × 0.25-mm areas of tubulointerstitial for each kidney.

For TGF-β1, MCP-1, and IL-1β staining, 4-μm sections of frozen tissues were fixed in acetone for 10 min at −20°C and then rinsed with PBS. Sections were then blocked with a 1.5% solution of serum and incubated with primary antibodies, hamster anti-mouse antibody against MCP-1 (clone 2H5, 1:50 dilution, BioSource International), rabbit polyclonal antibodies against IL-1β (1:100 dilution, Endogen, Woburn, MA), and TGF-β1 (1:150 dilution, Santa Cruz Biotechnol- ogy, Santa Cruz, CA), and then secondary antibodies as described above. Methyl green was used as a counterstain.

Statistical analysis. Continuous variables, expressed as means ± SE, were compared with ANOVA derived from general linear models. Pairwise comparisons of physiological data from weeks 4, 12, and 24 were performed by using the Student-Newman-Keuls procedure. Determinants of proteinuria, glomerulosclerosis, and tubulointerstitial injury were analyzed by using multivariable linear regression with stepwise variable selection. Multiple interaction terms were tested to evaluate whether the estimated effects of blood pressure and degree of proteinuria on glomerulosclerosis were uniform across treatment modality. Dependent variable distributions approximated the normal, and regression diagnostics showed no outliers. Repeated-measures ANOVA, factorial ANOVA, and paired t-tests were employed for other comparisons. For PCR data, which were not normally distributed, differences among multiple groups were assessed by using the Kruskal-Wallis test and those between two groups with the Mann-Whitney U-test. P < 0.05 were considered significant. Statistical analyses were conducted by using Statview 4.01 (Abacus Concepts, Berkley, CA) and SAS 6.08 (SAS Institute, Cary, NC).

RESULTS

Chronic studies. Mean body weight increased in all groups during the study, and no statistical differences in body weight developed between candesartan- and enalapril-treated rats over time in either the 12- or 24-wk sets. Sham-operated rats attained significantly greater body weight than partially nephrectomized rats in the pretreatment period and continued to main- tain significantly higher average body weight than enalapril-treated rats in the 24-wk set. In the 12-wk set only the difference between sham-operated and enalapril-treated rats in the pretreatment period was statistically significant. (Table 2).

Mean SBP increased in all groups after partial nephrectomy and did not differ statistically among the groups before initiation of therapy at week 5. Treatment with either candesartan or enalapril resulted in an initial fall in SBP to levels similar to those of sham-operated rats. SBP remained similar among treated groups and sham-operated rats over weeks 6-12 in both 12- and 24-wk sets. Thereafter, there was a gradual increase in SBP such that from 18 wk, SBP levels were statistically higher than the lowest values, observed at 8 wk, in both CSN_B and ENA_B rats. There were no statistically significant differences in SBP between treatment groups over time, in either 12- or 24-wk sets (mean differences: CSN_A vs. ENA_A = 11 mmHg, P = 0.26; CSN_B vs. ENA_B = 7 mmHg, P = 0.41). Sham-operated rats remained normotensive throughout the study (Figs. 2A and 3A). UprV increased after partial nephrectomy and was similar among the groups before initiation of therapy at week 5. In both candesartan- and enalapril-treated rats, UprV declined at first, but later increased progressively to reach lev- els approximately twofold those of pretreatment values and eight- to ninefold those of sham-operated rats at 24 wk. No statistically significant differences were observed in UprV between treatment groups over time, in either the 12- or 24-wk sets (mean differences: CSN_A vs. ENA_A = 8.8 mg/day, P = 0.16; CSN_B vs. ENA_B = 8.4 mg/day, P = 0.31). In sham-operated rats, mean UprV remained low, although a small increase was evident with time (Figs. 2B and 3B). UprV was directly corre- lated with SBP in combined data from CSN_B and ENA_B rats at 12 and 24 wk (r = 0.60 and 0.73, respectively; P < 0.0001 for both). There were still no differences in UprV between the treatment groups after adjusting for the effects of SBP (P = 0.50 and P = 0.70 at 12 and 24 wk, respectively). Furthermore, there was no effect of treatment group on the relationship between UprV and

Table 2. Body weights

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>WK5</td>
<td>6</td>
<td>250±2(5)</td>
<td>272±7(18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSN_A</td>
<td>11</td>
<td>260±2(9)</td>
<td>282±7(7)</td>
<td>306±4 (14)</td>
<td>312±2 (9)</td>
<td>322±10(24)</td>
<td>331±15(36)</td>
<td></td>
</tr>
<tr>
<td>ENA_A</td>
<td>11</td>
<td>253±5(16)</td>
<td>270±7(22)*</td>
<td>312±9 (28)</td>
<td>328±7 (24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham_A</td>
<td>6</td>
<td>261±3(6)</td>
<td>301±6(15)</td>
<td>322±10(24)</td>
<td>331±15(36)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSN_B</td>
<td>19</td>
<td>262±2(7)</td>
<td>282±3(14)†</td>
<td>309±4 (16)</td>
<td>331±5 (20)</td>
<td>347±4(16)</td>
<td>360±4(19)</td>
<td>360±7(30)</td>
</tr>
<tr>
<td>ENA_B</td>
<td>16</td>
<td>256±2(10)</td>
<td>278±3(13)†</td>
<td>309±4 (15)</td>
<td>327±4 (17)</td>
<td>344±5(19)</td>
<td>350±6(23)</td>
<td>359±6(24)‡</td>
</tr>
<tr>
<td>Sham_B</td>
<td>9</td>
<td>266±2(5)</td>
<td>298±2(20)</td>
<td>332±5 (14)</td>
<td>350±4 (13)</td>
<td>359±5 (16)</td>
<td>369±4(11)</td>
<td>373±5(16)</td>
</tr>
</tbody>
</table>

Values are means ± SE (SD) in (g). n, No. rats; CSN_A and CSN_B, candesartan-treated rats; ENA_A and ENA_B, enalapril-treated rats; Sham_A and Sham_B, sham-operated rats; WK5, rats killed after 5 wk of renal ablation. *P < 0.05 vs. Sham_A over wk 0–4. †P < 0.05 vs. Sham_B over wk 0–4. ‡P < 0.05 vs. Sham_B over wk 6–24.
SBP (interactive terms: $P = 0.78$ and $P = 0.30$ at 12 and 24 wk, respectively).

Remnant kidneys hypertrophied considerably in all groups such that the weight increased three to fourfold over baseline. There were no statistically significant differences in final remnant kidney weight among the groups. Analysis of remnant kidney weights, expressed as kidney weight-to-body weight ratio, yielded similar results (Table 3).

**Morphology.** Histological data are summarized in Table 4. At 5 wk after partial nephrectomy and before initiation of therapy, glomerulosclerosis was evident in a mean of 26 ± 6% of glomeruli (5WK rats). At 12 wk postsurgery, the extent of glomerulosclerosis in candesartan- and enalapril-treated rats was similar to that observed before treatment in the 5-wk group; more-
Moreover, there was no statistical difference in mean GS between CsnA and EnaA. At 24 wk after surgery, there was again no statistically significant difference in the mean GS of CsnB vs. EnaB rats. Comparison of combined CsnB and EnaB data with combined CsnA and EnaA data revealed a trend toward more extensive glomerulosclerosis at 24 vs. 12 wk (P = 0.06 by ANOVA). When these data are viewed in the context of data for untreated 5/6 nephrectomized controls at 12 wk, it is evident that both treatments slowed the progression of secondary FSGS such that the extent of glomerulosclerosis previously observed at 12 wk after 5/6 nephrectomy (GS = 43 ± 17 (SD) %), was attained only after 24 wk in CsnB and EnaB rats (Fig. 4). Minimal glomerulosclerosis was noted in sham-operated rats.

Tubulointerstitial injury showed similar patterns of change to those of glomerulosclerosis and was also not statistically different between CSN and ENA rats at 12 or 24 wk. There was a direct and highly significant correlation between GS and TIS in pooled data from CsnB and EnaB rats (r = 0.85; P < 0.001). As with glomerulosclerosis, minimal tubulointerstitial injury developed in sham-operated rats.

Multivariable analysis. Analysis of data from rats killed at 5 wk after surgery revealed statistically significant correlations between pretreatment UprV and GS (r = 0.87; P = 0.02) or TIS (r = 0.87; P = 0.02).

Table 3. Remnant kidney weight and kidney weight-to-body weight ratio

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Remnant Kidney Wt</th>
<th>Remnant Kidney Wt-to-Body Wt Ratio, g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline Final</td>
<td>Final</td>
</tr>
<tr>
<td>WK5</td>
<td>6</td>
<td>0.29 ± 0.01(0.02)*</td>
<td>1.16 ± 0.08(0.20)</td>
</tr>
<tr>
<td>CsnA</td>
<td>11</td>
<td>0.33 ± 0.01(0.03)</td>
<td>1.28 ± 0.03(0.09)</td>
</tr>
<tr>
<td>EnaA</td>
<td>11</td>
<td>0.35 ± 0.01(0.03)</td>
<td>1.25 ± 0.07(0.22)</td>
</tr>
<tr>
<td>CsnB</td>
<td>19</td>
<td>0.36 ± 0.01(0.02)</td>
<td>1.43 ± 0.06(0.25)</td>
</tr>
<tr>
<td>EnaB</td>
<td>16</td>
<td>0.35 ± 0.01(0.03)</td>
<td>1.39 ± 0.07(0.29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.22 ± 0.04(0.09)*</td>
<td>4.25 ± 0.24(0.59)</td>
</tr>
</tbody>
</table>

Values are means ± SE (SD) in (g), n, No. of rats. Baseline kidney weights are an estimate calculated as one-third of the weight of the kidney resected at the time of initial surgery. Final remnant kidney weights (or kidney weight-to-body weight ratios) were significantly greater than baseline in all groups (P < 0.0001). There were no statistically significant differences in final remnant kidney weight (or kidney weight-to-body weight ratio) among the groups. *P < 0.05 vs. all treatment groups.

There were no statistically significant correlations between pretreatment SBP and GS or TIS.

At 24 wk, direct and highly significant correlations were evident between SBP and GS (r = 0.81; P < 0.0001) (Fig. 5A). Similarly, UprV was highly correlated with GS (r = 0.86; P < 0.0001) (Fig. 5B). By contrast, there was no effect of treatment group on GS at 24 wk (P = 0.9). Stepwise multiple linear regression analysis with GS as the dependent variable and SBP, UprV, and remnant kidney weight-to-body weight ratio as independent variables, entered only SBP and UprV into the model. These variables together accounted for 72% of the variance in glomerulosclerosis observed at 24 wk. The magnitude of the effects of SBP and UprV as determinants of glomerulosclerosis was such that a 10-mmHg change in SBP or a 10 mg/day change in UprV was each associated with a change of three percentage points in GS at 24 wk.

TIS at 24 wk also correlated with SBP and UprV (r = 0.78 and 0.69, respectively; P < 0.0001 for both). Step-

Table 4. Histological analysis of remnant kidney tissue

<table>
<thead>
<tr>
<th>Group</th>
<th>Time Postsurgery, wks</th>
<th>Glomerulosclerosis Score, %</th>
<th>Tubulointerstitial Score (0–3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WK5</td>
<td>6</td>
<td>25.5 ± 6.4(16)</td>
<td>0.6 ± 0.3(0.8)</td>
</tr>
<tr>
<td>CsnA</td>
<td>11</td>
<td>32.1 ± 4.2(13.8)</td>
<td>0.9 ± 0.1(0.4)</td>
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<tr>
<td>CsnB</td>
<td>19</td>
<td>41.5 ± 6.5(28.3)</td>
<td>1.3 ± 0.3(1.1)</td>
</tr>
<tr>
<td>EnaA</td>
<td>11</td>
<td>29.2 ± 4.6(15.3)</td>
<td>0.9 ± 0.2(0.8)</td>
</tr>
<tr>
<td>EnaB</td>
<td>16</td>
<td>41.8 ± 4.4(17.5)</td>
<td>1.2 ± 0.2(0.7)</td>
</tr>
<tr>
<td>ShamA</td>
<td>6</td>
<td>0.2 ± 0.1(0.4)</td>
<td>0.0 ± 0.0(0.0)</td>
</tr>
<tr>
<td>ShamB</td>
<td>9</td>
<td>2.8 ± 0.4(1.2)</td>
<td>0.0 ± 0.0(0.0)</td>
</tr>
</tbody>
</table>

Values are means ± SE (SD). n, No. of rats. TIS from 0 to 3 is based on estimated percentage of abnormal tissue: 0, <20; 20–50, or >50%. There were no statistically significant differences in glomerulosclerosis score (GS) or tubulointerstitial score (TIS) between Csn and Ena rats in either the 12- or 24-wk set. A trend toward an increase in GS from 12 to 24 wk in combined data from Csn and Ena rats was not statistically significant (P = 0.06).

Fig. 4. GS at different time points in untreated (5WK; solid bar), Csn (hatched bars), and Ena rats (stippled bars). Horizontal lines indicate means ± SD for GS at 12 wk after 5/6 nephrectomy in 22 untreated control rats from previous studies.
wise multiple linear regression analysis with TIS as the dependent variable and the same independent variables as above entered only SBP into the model.

**Competitive RT-PCR.** Mean levels of β-actin mRNA were similar among groups for each set of PCR reactions, confirming that starting concentrations of cDNA were similar and not subject to systematic error. Renal cortex mRNA levels for TGF-β1 and MCP-1 in 5/6 nephrectomized rats before treatment were about twofold higher than those of sham-operated rats. At 12 wk after surgery, TGF-β1 and MCP-1 mRNA levels were similar to pretreatment 5WK values in both CsnB and EnaB rats and were significantly higher than in ShamA rats. For IL-1β, mRNA levels were similar in 5WK and ShamA rats. At 12 wk, IL-1β mRNA levels in both CsnB and EnaB rats were similar to those of ShamA rats (Fig. 6). There were no statistically significant differences in mRNA levels for any of the genes examined between CSN and ENA rats at either 12 or 24 wk.

Because there were no differences between candesartan- and enalapril-treated rats with respect to any of the parameters determined in this study, data from both groups were pooled for further analysis. In this combined group, strong and highly statistically signif-

Fig. 5. A: scatterplot for GS vs. SBP at 24 wk in CsnA (■; dashed line, regression line) and EnaA (○; dotted line, regression line) rats. Linear regression equation for combined data (solid line): $y = -34.5 + 0.52x$; $r^2 = 0.65$. B: scatterplot for GS vs. UprV at 24 wk in in CsnA (■; dashed line, regression line) and EnaA (○; dotted line, regression line) rats. Linear regression equation for combined data (solid line): $y = -6.5 + 0.51x$; $r^2 = 0.74$.

Fig. 6. Renal cortex mRNA levels for TGF-β1 (top), monocyte chemoattractant protein (MCP)-1 (middle), and interleukin (IL)-1β (bottom; expressed as a ratio to the mean 5-wk untreated value) in 5/6 nephrectomized rats receiving no treatment (5WK; solid bars), Csn (hatched bars), Ena (stippled bars), and sham-operated rats (Sham; open bars). *$P < 0.05$ vs. 5WK. †$P < 0.05$ vs. Sham. §$P = 0.05$ vs. Sham.
significant correlations were evident between 24-wk renal cortex mRNA levels for TGF-β1, MCP-1, and IL-1β and the extent of FSGS (Fig. 7) or TIF (Table 5). Somewhat weaker but nevertheless statistically significant correlations were evident among TGF-β1, MCP-1, and IL-1β, and SBP or UprV (Table 5). Analysis of pooled CsnA and EnaA data revealed only weak or absent correlations among these parameters at 12 wk after surgery (data not shown).

Immunohistology. Immunohistology confirmed that the increases in gene expression detected by competitive RT-PCR were accompanied by qualitative increases in expression of the gene product and localized the protein expression within the renal cortex. Negative controls, in which no primary antibody was used, showed minimal staining of tubules and no glomerular staining. For TGF-β1, kidneys from sham-operated rats exhibited moderate staining of tubules and negative staining of glomeruli. Among 5WK rats, positive TGF-β1 staining of both tubules and glomeruli was observed. Patterns of staining similar to those of 5WK rats were observed among rats in both treatment groups, with particularly strong staining seen in areas of segmental glomerulosclerosis (Fig. 8, A–C). MCP-1 staining was limited to minimal positivity of tubule cells in sham-operated rats. Positive MCP-1 staining of tubules and glomeruli was observed in 5WK rats and among rats from both treatment groups (Fig. 8, D–F). Positive staining for IL-1β was localized mainly to tubule cells in specimens from all groups. However, among 5WK rats and rats from the treatment groups, focal areas of positive staining for IL-1β were observed in some glomeruli (Fig. 8, G–I). (This observation is consistent with staining of individual cells within glomeruli but, due to the limitations of histology performed on frozen tissue sections, this could not be confirmed.)

Macrophage infiltration. Extensive infiltration of glomeruli and remnant kidney interstitium by macrophages was evident before initiation of therapy at 5 wk after surgery. Whereas macrophages were virtually absent from the kidneys of sham-operated rats, a mean of 5.7 ± 0.22 macrophage/glomerular profile and 6.6 ± 0.19 macrophages/0.0625-mm² area of interstitium were observed in 5WK rats. Treatment with candesartan or enalapril was associated with two- to fivefold reductions in glomerular macrophage infiltration and an approximately fourfold reduction in interstitial macrophages at 12 wk after surgery. Nevertheless, the extent of macrophage infiltration of both glomeruli and interstitium remained significantly higher in treated rats vs. sham-operated rats at 12 and 24 wk. Glomerular macrophage counts were slightly, albeit significantly, higher in enalapril- vs. candesartan-treated rats at 12 and 24 wk. There was no difference in interstitial macrophage counts among treatment groups at either time point (Fig. 9).

DISCUSSION

These results show that, even when started after the onset of renal injury, the renoprotective effects of can-

diesartan or enalapril were observed in 5WK rats and among rats from both treatment groups (Fig. 8, D–F). Positive staining for IL-1β was localized mainly to tubule cells in specimens from all groups. However, among 5WK rats and rats from the treatment groups, focal areas of positive staining for IL-1β were observed in some glomeruli (Fig. 8, G–I). (This observation is consistent with staining of individual cells within glomeruli but, due to the limitations of histology performed on frozen tissue sections, this could not be confirmed.)

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DISCUSSION

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Table 5. Correlation coefficients for renal cortex cytokine mRNA levels vs. measures of renal injury in a combined group of Csn and Ena rats at 24 wk after 5/6 nephrectomy

<table>
<thead>
<tr>
<th></th>
<th>SBP</th>
<th>UprV</th>
<th>GS</th>
<th>TIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>0.67*</td>
<td>0.77*</td>
<td>0.81*</td>
<td>0.78*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.57*</td>
<td>0.46†</td>
<td>0.57*</td>
<td>0.56*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.65*</td>
<td>0.57*</td>
<td>0.70*</td>
<td>0.73*</td>
</tr>
</tbody>
</table>

SBP, systolic blood pressure; UprV, urinary protein excretion rate.

*P < 0.0005, †P < 0.01.
desartan are equivalent to those of enalapril when dosing is adjusted to achieve similar levels of SBP control. Comparison with data for untreated rats from previous studies in this model shows that delayed treatment with either ACEI or AT₁ RA, appears to slow the rate of progression of glomerulosclerosis by about one-half, an effect reminiscent of that achieved in clinical trials (14, 27, 30). Our study differs from the previous study comparing delayed therapy with ACEI and AT₁ RA in the 5/6 nephrectomy model by virtue of its longer duration and increased statistical power (34). Furthermore, 5/6 nephrectomy was achieved by surgical excision of renal mass in the former study whereas we performed infarction of 2/3 of the left kidney, a model resulting in more severe renal injury and greater activation of the RAS (12, 54) and therefore more likely to expose subtle differences in efficacy between ACEI and AT₁ RA. Nevertheless, we detected no differences among the treatment groups with respect to any of the markers of renal injury examined in this study (Uₚᵥ, GS, and TIS) or in the remnant kidney levels of cytokine gene expression. Moreover, no tendencies for differences to emerge were detected even over a prolonged observation period. The extent of hypertrophy of the remnant kidney also was similar between ACEI- and AT₁ RA-treated rats. On the basis of the differences in the mechanisms, whereby ACEI and AT₁ RA inhibit the RAS, it has been suggested that AT₁ RA may have therapeutic benefits over ACEI because they inhibit the actions of ANG II formed by other serine proteases in the presence of ACEI or because of the antihypertensive and antiproliferative effects that may result from stimulation of AT₂ receptors by elevated ANG II levels during blockade of AT₁ receptors. On the other hand, it has been suggested that at least some of the therapeutic benefit
of ACEI results from elevation of bradykinin levels, which is not present during AT1RA treatment (51). Our findings strongly suggest that although they inhibit the RAS at different levels, both ACEI and AT1RA exert their beneficial effects predominantly by inhibiting the effects of ANG II mediated by AT1 receptors. This conclusion is consistent with the findings of previous studies showing that the elevated bradykinin levels associated with ACEI (2, 24, 33), or the AT2 receptor stimulation that results indirectly from blockade of the AT1 receptor (9), do not appear to play significant renoprotective roles.

The success of RAS inhibitors in reducing renal injury in nonhypertensive models of renal disease (17) and preserving renal function in normotensive patients (27) suggests that functional integrity of the RAS may itself be regarded as a risk factor for CRD progression. On the other hand, RAS inhibitors are highly effective antihypertensive and antiproteinuric agents. It is not yet clear to what extent blood pressure control retains its importance as a renoprotective measure in the context of RAS inhibition. Nor is it clear whether reduction of proteinuria merely reflects blood pressure reduction or if lower levels of protein excretion at a given level of systemic blood pressure are associated with additive renal protective effects. A major finding of this study, therefore, is the demonstration by linear regression techniques that systolic blood pressure and urinary protein excretion are independent determinants of glomerulosclerosis in rats receiving inhibitors of the RAS after extensive renal ablation. Together, SBP and UrV accounted for 72% of the variance in GS, implying that they represent the major determinants of glomerulosclerosis in this model.

Although it is not possible to exclude from these data the possibility that higher levels of blood pressure merely reflect greater severity of renal injury, it is true to say that failure to control blood pressure optimally in this model was associated with failure to achieve renal protection. Furthermore, the lack of any correlation between the level of SBP and severity of renal injury at an early time point, before the initiation of treatment, argues in favor of a direct effect of blood pressure on renal disease progression over time. Using radiotelemetry, Bidani et al. (6) also found a strong correlation ($r = 0.88$) between mean SBP and glomerulosclerosis in untreated 5/6 nephrectomized rats. In keeping with our observations, the correlation between SBP and glomerular injury was much weaker during the first 2 wk after injury. The renoprotective effect of lowering blood pressure has been clearly established in clinical studies of CRD (20, 22, 23, 32, 37, 39). In diabetic patients treated with ACEI, lower target levels of blood pressure control have been shown to result in greater renoprotective effects in one randomized trial (28). Our data are therefore consistent with this clinical evidence that the level of blood pressure control remains an important determinant of progressive renal injury in CRD during treatment with inhibitors of the RAS. It should be remembered that micropuncture studies in 5/6 nephrectomized rats suggest that $P_{gc}$, rather than systemic blood pressure per se, is the critical determinant of renal injury (4, 50) and that ACEI (4) and AT1RA (26, 29) reduce both systemic and glomerular capillary pressures.

Proteinuria has traditionally been regarded merely as a marker of glomerular injury. Recent clinical studies, however, report that proteinuria may also be an independent epidemiological risk factor of CRD progression (8, 14, 39). Furthermore, treatments that reduce proteinuria also slow the progression of CRD (14, 27, 30), and a reduction in proteinuria, independent of blood pressure, was associated with slower progression of CRD in the MDRD study (39). Together, these findings raise the possibility that proteinuria per se exacerbates renal injury. Experimental observations suggest mechanisms whereby filtered proteins may contribute to renal damage. Exposure of mesangial cells to plasma lipoproteins in vitro results in prolifer-
ation, expression of proinflammatory cytokines, and synthesis and elaboration of extracellular matrix protein, all of which may contribute to the pathogenesis of glomerulosclerosis (15, 44). More recently, culture of tubular epithelial cells in the presence of a variety of plasma proteins has been shown to induce production of proinflammatory cytokines and extracellular matrix proteins (1, 56, 59, 61), responses that may contribute to tubulointerstitial fibrosis. In vivo, proteinuria induced by protein overload was associated with renal expression of cell adhesion molecules and chemokat-
tants, resulting in interstitial inflammation and fibrosis (13). A meta-analysis of 57 animal studies, including various models of renal disease, reported consistent positive associations between the level of protein- or albuminuria and the severity of glomerulosclerosis (mean weighted correlation coefficients \( r = 0.82 \) and 0.76) (38). We have confirmed that a direct correlation exists between proteinuria and glomerular injury in 5/6 nephrectomized rats receiving RAS inhibitors \( (r = 0.86) \), independent of the level of blood pressure. This implies that at a given level of blood pressure, rats with higher levels of proteinuria can be expected to develop more severe renal injury, a conclusion similar to those supported by clinical studies (14, 39). Although these data do not prove that proteinuria per se contributes to renal injury, they do reveal the extent to which the renoprotective effects of ACEI and AT\(_1\)RA are related to their antiproteinuric effects.

The detection of inflammatory and profibrotic gene induction and macrophage infiltration in the remnant kidney supports the notion that inflammatory processes may contribute to the progressive renal injury and fibrosis that follows 5/6 nephrectomy. Among possible mechanisms whereby proinflammatory gene expression may be stimulated in the remnant kidney are exposure of glomerular cells to mechanical stresses resulting from augmented glomerular hemodynamics (35, 43, 49), direct effects of ANG II (18, 25), and exposure of tubule epithelial cells to abnormal amounts of filtered protein (13, 52, 56, 59). Thus components of an inflammatory process may be induced in the remnant kidney in the absence of classic immune stimuli. Previous studies from this and other laborato-
ries have shown that the protection from progressive renal injury afforded by ACEI or AT\(_1\)RA treatment initiated early after 5/6 nephrectomy is associated with normalization of \( P_{\text{Ge}} \) (4, 29), suppression of proinflam-
matory gene induction to levels similar to those of sham-operated rats, and inhibition of renal macrophage infiltration to levels only slightly greater than sham (53, 57). In this study, we observed that when treatment was delayed until 5 wk after 5/6 nephrectomy, a time point when remnant kidney mRNA levels for TGF-\( \beta \)1 and MCP-1 are known to be upregulated (53), ACEI or AT\(_1\)RA did not suppress the expression of these two cytokines to normal levels. At 12 wk post-surgery mRNA levels for TGF-\( \beta \)1 and MCP-1 were similar to those observed before the initiation of treat-
ment and remained significantly higher than those of sham-operated rats. At 24 wk after surgery, TGF-\( \beta \)1 mRNA levels were significantly lower than pretreatment values but remained significantly higher than sham levels, and MCP-1 mRNA levels remained at pretreatment values. Thus failure of suppression of the TGF-\( \beta \)1 and MCP-1 responses at 12 wk, when renal injury had not yet progressed beyond that observed before the initiation of treatment, was associated with slow progression of renal injury despite likely amelio-
ration of adverse glomerular hemodynamic factors. IL-1\( \beta \) mRNA levels were not elevated in pretreatment vs. sham-operated rats. This is consistent with previ-
ous observations from this laboratory that IL-1\( \beta \) induction was not apparent until 8 wk after 5/6 nephrectomy (53). The trend toward higher IL-1\( \beta \) mRNA levels in both treatment groups vs. sham at 12 wk suggests that failure of suppression of this gene, a product of activated macrophages, may also be associated with sub-
sequent progression of injury. The strong correlations observed between the extent of renal injury at 24 wk (as measured by either GS or TIS) and mRNA levels for TGF-\( \beta \)1, MCP-1, and IL-1\( \beta \) further support the hypo-
thesis that upregulation of these proinflammatory and profibrotic genes contributes to progressive renal injury.

Together, these observations in remnant kidneys indicate 1) that incomplete suppression of proinflam-
matory gene expression with ACEI or AT\(_1\)RA treat-
ment is associated with failure to arrest the progres-
sion of renal injury and 2) that the extent of progression is directly correlated with the level of gene expression. It should be stressed that these observa-
tions were made in rats receiving chronic treatment at doses of ACEI or AT\(_1\)RA with documented success in normalizing \( P_{\text{Ge}} \) even when initiated after the onset of renal injury in this model (16, 26, 31). This implies that the process of renal injury initiated by glomerular capillary hypertension, and the direct or indirect ef-
effects of ANG II, eventually may be sustained more by autonomous cellular and molecular factors, and be-
come less dependent on the initiating factors. This notion is consistent with the observations of Ichikawa and others (16) that in glomeruli with severe estab-
lished injury, treatment with enalapril did not prevent further progression to global sclerosis (16). Alterna-
tively, it remains possible that, in the face of existing renal injury, treatment with ACEI or AT\(_1\)RA did not completely normalize \( P_{\text{Ge}} \) in the long term or achieve total blockade of the RAS. In keeping with suggestions by other authors (7), these findings imply that patients in whom progression of chronic renal injury persists, albeit slowly, during RAS blockade, may benefit from additional therapy targeting the effects of inflamma-
tory and profibrotic cytokine gene expression.

Conclusions. We have provided further evidence that, despite differences in their site of inhibition of the RAS, ACEI and AT\(_1\)RA have equivalent renal protec-
tive effects in 5/6 nephrectomized rats. Furthermore, in the context of RAS inhibition, the levels of both blood pressure and urinary protein excretion rates serve as major and independent determinants of glomeruloscle-
rosis. In addition, the incomplete suppression of in-
flammatory and profibrotic gene expression observed when ACEI or AT1-RA treatment is started after the onset of renal injury may contribute to the slow progression of CRD during RAS inhibition in this model. Although prospective clinical trials are required to confirm these findings in humans, it would seem reasonable to conclude that normalization of blood pressure and maximal reduction of proteinuria should be important therapeutic goals in clinical strategies aiming to achieve renal protection with RAS inhibitors. Further studies are required to evaluate whether additional therapy targeting the effects of inflammatory and profibrotic cytokine gene expression may further slow the rate of CRD progression.

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REFERENCES
MECHANISMS OF RENOPROTECTION DURING RAS BLOCKADE


