Long-term Infusion of Kallikrein Attenuates Renal Injury in Dahl Salt-Sensitive Rats

Yoshio Uehara, Nobuhito Hirawa, Yukari Kawabata, Tomohiko Suzuki, Noriko Ohshima, Kazuo Oka, Toshio Ikeda, Atsuo Goto, Teruhiko Toyo-oka, Kazuyuki Kizuki, Masao Omata

Abstract We investigated whether long-term infusion of kallikrein would attenuate renal injury in salt-induced hypertension in Dahl salt-sensitive rats. A subdepressor dose of purified rat urinary kallikrein (700 ng/d IV) was infused by osmotic minipump for 4 weeks in male Dahl salt-sensitive rats fed a high salt (2% NaCl) diet. This dose did not affect the time-dependent elevation of blood pressure; however, urinary protein excretion was significantly decreased, and glomerular filtration rate was increased. These beneficial effects were reflected morphologically by an attenuation of the glomerulosclerotic lesions and tubular injury seen in the hypertensive Dahl salt-sensitive rats. Kallikrein infusion increased urinary excretion of bradykinin and stimulated excretion of cyclic cGMP, suggesting that the kallikrein-kinin-prostaglandin and nitric oxide axes were enhanced by rat urinary kallikrein infusion. The alterations induced by kallikrein infusion were potentiated by the concomitant administration of the angiotensin-converting enzyme inhibitor alacepril. These studies indicated that long-term replacement with rat tissue kallikrein attenuates renal injury in hypertensive Dahl salt-sensitive rats.

(Angiotensin-converting enzyme (ACE) inhibitors affect both the renin-angiotensin and kallikrein-kinin-prostaglandin systems, inhibiting different catalytic sites on the same ACE. The cardiovascular protection induced by ACE inhibitors is primarily due to inhibition of the renin-angiotensin system. Recent advances in vascular biology suggest that the vasodepressor system participates in the progression or regression of cardiovascular injury. Vasodepressor cyclicicosanoid directly delays the transition from the resting G0/G1 to DNA-synthetic (S) period progression in the proliferative cycle of vascular smooth muscle cells. Moreover, enhancement of the endogenous production of prostacyclin attenuates vascular and renal injury in salt-induced hypertension in Dahl salt-sensitive (Dahl S) rats, a genetic model for salt-induced hypertension in Dahl S rats. Kinins stimulate the formation of nitric oxide, which also retards vascular smooth muscle cell proliferation. The activity of the kallikrein-kinin-prostaglandin system is reduced in the kidneys of Dahl S rats. Kinins stimulate the formation of nitric oxide, which also retards vascular smooth muscle cell proliferation. The mechanism is not certain. The close relation between the kallikrein-kinin-prostaglandin system and the genesis of salt-induced hypertension in Dahl S rats led us to propose that the kallikrein-kinin-prostaglandin axis contributes to the susceptibility of Dahl S rats to hypertensive injury. If this assumption is true, then replacement with rat urinary kallikrein (RUK) may activate the vasodepressor system and thereby attenuate the target-organ injury seen in Dahl S rats.

To test this hypothesis, we infused RUK continuously into the systemic circulation of Dahl S rats for 4 weeks and investigated whether this replacement attenuates the renal injury seen in Dahl S rats with salt-induced hypertension.

Methods

Preparation of RUK

RUK was purified from 3000 mL of pooled urine from Sprague-Dawley rats according to the established method. The crude enzyme fraction was precipitated by ammonium sulfate solution (final concentration, 70%). The purification procedure was followed by a series of ion-exchange chromatography steps using DEAE-Sephadex (Pharmacia LKB Biotechnology) and DEAE-Sephrose CL-6B (Pharmacia LKB), followed by gel permeation chromatography using Sephadex G-75 (Pharmacia LKB) and hydrophobic chromatography using phenyl-Sepharose CL-4B (Pharmacia LKB), and finally by ion-exchange DEAE-Sephadex A-50 chromatography (Pharmacia LKB). The purified fraction was passed through a membrane sieve (Millex-HA ultrafiltering filter, 0.45 μm, Japan Millipore Ltd) lyophilized, and stored at −70°C in sterile conditions.

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A single band was detected in lane 1 and 2 with RUK, but not in lane 3 without RUK enzyme (b, negative control). Upper end is cathode; lower end is anode (+).

RUK purity was assessed with polyacrylamide slab gel electrophoresis (PAGE), sodium dodecyl sulfate (SDS)-PAGE, and Western blot analysis. The enzyme was dissolved in 50 mmol/L Tris chloride (pH 6.8) containing 10% (vol/vol) glycerol, 1 mmol/L diithiothreitol, and 0.0025% (wt/vol) bromophenol blue with or without 2% (wt/vol) SDS. Samples were applied to a 7.5% polyacrylamide slab gel and analyzed with a PROTEAN II Slab Cell (Bio-Rad) and MultiDrive XL power supply unit (Pharmacia LKB). Proteins were stained with 0.1% Coomassie brilliant blue R-100 (Bio-Rad) in fixative (40% methanol and 10% acetic acid). The molecular weight of the band of interest was determined by referring to the relative mobility of molecular weight standards.

Purified RUK exhibited a specific activity of 10 arbitrary units per milligram protein. This enzyme hydrolyzed rat kininogens and generated bradykinin, as determined by a specific radioimmunoassay kit, Bio-Rad) (Fig 1b). Its molecular weight was estimated according to methods described in the text. Eight micrograms of purified RUK was applied onto lane 1 and 16 ^g onto lane 2 (a).

Blood pressure was measured every week by the tail-cuff method of Friedman and Freed. At the end of the experiment, arterial pressure was determined directly by the telemetric method through a cannula inserted into the abdominal aorta with rats under thiobutabarbital anesthesia (25 mg/kg body wt) (BYK-Gulden). A 24-hour urine specimen was collected on the last day of the experiment. For histological investigation, the right kidney was obtained with rats under thiobutabarbital anesthesia. Then whole blood samples and organs of interest were obtained after rats were killed under anesthesia.

Renal Function
Plasma and urinary creatinine levels were measured by a creatinine analyzer (model TDX, Dainabot Co, Ltd). Plasma electrolyte levels were measured with a flame photometer (AutoCal model 643, Instrumentation Laboratories, Inc). Urinary protein concentration was measured by the sulfosalicylic acid method. Urinary albumin excretion was determined with a standard enzyme-linked immunosorbent assay method (Neph-rat kit; Funakoshi Co, Ltd). N-Acetyl-β-d-glucosaminidase (NAG) activity in urine was measured using sodio-m-cresolsulphonphthalaeinyl NAG as the substrate (NAG assay kit, Shionogi Pharmaceutical Co).

Radioimmunoassay of Urinary Eicosanoids
Urinary prostaglandin excretion was measured by extraction and radioimmunoassay methods as described previously. In brief, an aliquot of collected urine, acidified to pH 3.5 with 0.2 mol/L citrate-buffered solution, was applied to a Sep-Pak C18 cartridge (Waters Associates). The cartridge had been equilibrated with 20% ethanol in 0.2 mol/L citrate-buffered solution (vol/vol) at pH 3.5. The cartridge was washed successively with 20 mL of distilled water, 20 mL of 20% chloroform in n-hexane (vol/vol), and 20 mL of absolute n-hexane. Finally, prostaglandins were eluted with 3 mL of methyl formate. The extracted prostaglandins were evaporated to dryness and dissolved in 1 mL of 50 mmol/L phosphate-buffered solution for radioimmunoassay. The recovery rate averaged 88.9±0.3%.

The assay mixture consisted of 0.1 mL [3H]prostaglandin (10 disintegrations per minute), 0.1 mL diluted sample or standard solution, and 0.1 mL diluted antibody solution. The assay mixture was incubated at 4°C for 24 hours. To separate the bound from the free form, 0.1 mL of a dextran-coated charcoal solution (2.5% charcoal and 0.25% dextran) was added, and the mixture was spun immediately at 1000g at 4°C for 5 minutes. The radioactivity of the bound form was counted with an automatic liquid scintillation counter.

In Vivo Experiment
Dahl S rats were obtained from Seiwa Experimental Animal Laboratory Co Ltd (Fukuoka, Japan). These rats originated from the colony kept at Mollegaards Avislaboratorium Aps (Ejby, Denmark). They had been fed a low salt (0.3% NaCl) diet after weaning. At the start of our study, thirty-six 8-week-old Dahl S rats were given a high salt (2% NaCl) diet. After 6 weeks, the rats were assigned randomly to the following four groups: (1) 9 rats were continued on a high salt diet alone (Dahl-2% control); (2) 7 rats were fed a high salt diet and given purified RUK at 700 ng/d (Dahl-RUK); (3) 11 rats were fed a high salt diet and given the ACE inhibitor alacepril [1-(S)-3-acetyltio-bio-2-methylpropionyl]-L-propyl-L-phenylalanine] (10 mg/kg body wt per day) (Dahl-ALCP); and (4) 9 rats were fed a high salt diet and given both RUK (700 ng/d) and alacepril (10 mg/kg body wt per day) (Dahl-RUK+ALCP). Alacepril was administered orally every day for 4 weeks. For a normotensive control group, five 8-week-old Dahl S rats were fed a low salt (0.3% NaCl) diet for 10 weeks. Water was given ad libitum during the study. In rats given RUK, kallikrein was infused into the right jugular vein with 14-day osmotic minipumps (model 2002, Alzet Corp). The same volume of vehicle (saline solution) was infused into the right jugular vein of Dahl-2% control and Dahl-ALCP rats. The pumps were changed every 2 weeks.

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The antisera were produced in our laboratory. Immunization was repeated every 2 weeks for 4 months. The dilution titer of anti-prostaglandin (PG) E₂ antiserum was 1:1×10⁴, and the detection limit (10% displacement of maximal binding) was 1.0 pg per tube. The cross-reactivity was 0.02% with 6-keto-PGF₁α, 0.01% with PGE₂, 0.005% with thromboxane B₂ (TXB₂), and less than 0.001% with arachidionate. The dilution titer of anti-TX-B₂ antiserum was 1:1.5×10⁵, and the detection limit was 2.5 pg per tube. The cross-reactivity was 0.4% with PGE₂, 0.2% with PGE₁a, 0.1% with PGI₂, 0.01% with TXB₂, and less than 0.001% with arachidonic acid. The dilution titer of anti-TXB₂ antibody was 1:1.5×10⁴, and the detection limit was 1 pg per tube. The cross-reactivity was 0.005% with 6-keto-PGF₁α, 0.007% with PGE₂, 0.02% with PGF₂α, 0.06% with PGD₂, and less than 0.0001% with arachidonic acid. The dilution titer of anti-PGF₂ antibody was 1:1×10⁴, and the detection limit was 1.0 pg per tube. The cross-reactivity was 0.09% with 6-keto-PGF₁α, 0.004% with PGD₂, 0.007% with PGE₁a, and less than 0.0001% with arachidonic acid.

**Radioimmunoassay of Urinary Bradykinin**

Urinary bradykinin levels were determined by a direct radioimmunoassay, as described by Shimamoto et al. briefly, 0.1 mL of 10⁻³-fold diluted urine or an appropriate standard bradykinin solution, 0.1 mL of 125I-tyrosyl-bradykinin, and 0.1 mL of 2×10⁵ diluted specific anti-bradykinin antibody were mixed and incubated at 4°C for 24 hours. To separate bound and free bradykinin, 0.3 mL of 1% human globulin solution was added as a carrier protein. The bound form of 125I-bradykinin was precipitated by addition of 1.0 mL of 25% polyethylene glycol solution. The mixture was vigorously vortexed and spun at 3000 rpm for 20 minutes at 4°C; the supernatant was removed by aspiration; and radioactivity in the precipitate was determined by an automatic gamma counter. The antibody used in the present study was raised by Shimamoto et al. briefly, a linear standard curve was obtained at concentrations ranging from 1 to 100 pg per tube. This antibody did not cross-react with arginine vasopressin, angiotensin II, or kinorogen.

**Radioimmunoassay of Cyclic GMP**

Urinary cyclic GMP (cGMP) levels were determined by direct radioimmunoassay (125I-RIA Kit, New England Nuclear-Daiichi Kagaku Chemicals Co., Ltd). Diluted (10⁻³-fold) urine or standard solution (0.1 mL), antibody solution (0.1 mL; first and second antibody complex), and 125I-cGMP (0.1 mL, 10⁴ dpm) were incubated at 4°C for 24 hours. The mixture was spun at 4°C for 30 minutes. After the supernatant was discarded, radioactivity in the precipitate was determined by an automatic gamma counter.

**Histological Investigation**

Half of each kidney was immediately fixed in 3.5% formalin, and sagittal slices were cut and embedded in paraffin. Sections 2 μm thick were cut and stained with hematoxylin and eosin, periodic acid-methenamine silver, and periodic acid–Schiff stains. All sections were evaluated by an independent investigator who had no prior knowledge of the group to which each rat belonged. More than 100 glomeruli in each specimen were examined. Glomerular sclerotic lesions were intensely stained by periodic acid–Schiff. The severity of these lesions was graded according to the percentage of the glomeruli involved: 0, no lesions (group 0); 1+, mild or intimal hyperplasia or necrosis with luminal narrowing (group 1); 2+, medial necrosis with inflammatory cell infiltration (group II); and 3+, medial and intimal hyperplasia and/or medial necrosis accompanied by inflammatory cell infiltration and thrombus formation (group III) (Fig. 1). An overall arterial injury score was obtained by multiplying the severity score (0 to 3+) by the percentage of arteries displaying the same degree of injury and summing these scores.

Tubular damage was scored according to a modification of the method of Rosen et al. Morin morphological alterations in the inner stripes and medullary rays were semiquantitatively evaluated and graded from 0 to 4+ as follows: 0, no lesions; 1+, very mild focal tubular dilation; 2+, increased number of dilated tubules associated with interstitial widening; 3+, fairly extensive dilation of tubules with cyst formation and interstitial widening; and 4+, complete atrophy of tubules. Tissue injuries in the medullary ray and inner stripe were scored separately for each section examined.

**Reagents**

All reagents were of analytical grade. Alacepril was donated by Dainippon Pharmaceutical Co., Ltd. Eicosanoids were donated by Ono Pharmaceutical Co., Ltd. Radioactive materials were purchased from New England Nuclear (Dai-ichi Chemicals Co., Ltd).

**Statistical Analysis**

Results are expressed as mean±SEM. Differences were analyzed by Student’s t test, one-way ANOVA, two-way ANOVA, and the χ² test for independence. A value of P<.05 was accepted as statistically significant.

**Results**

**Blood pressure in Dahl S rats in a time-dependent fashion.** Prolonged RUK infusion did not influence
his blood pressure elevation. After 4 weeks of treatment, the ACE inhibitor alacepril tended to attenuate the development of hypertension (195±5 versus 209±5 mm Hg, .05<P<.1). However, the combination of the ACE inhibitor with a subdepressor dose of RUK significantly potentiated the blood pressure reduction, and the difference became significant (209±5 versus 179±4 mm Hg, P<.0005).

At the end of the experimental period, we directly measured arterial blood pressure through a cannula placed in the abdominal aorta. There were no differences in arterial blood pressure between high salt control rats and rats given RUK alone (Table 1). Alacepril decreased systolic blood pressure by 12%. Addition of RUK decreased blood pressure by 19%. Dahl S rats fed a low salt diet had normal arterial pressures throughout the experiment.

As seen in Table 2, body weight did not differ among the various groups. Heart and aortic weights were increased in the high salt control group compared with normotensive 0.3% control rats. These parameters did not change with infusion of RUK alone. In the alacepril group, there was a trend toward reduced cardiac hypertrophy. This regression was more apparent in rats given simultaneous RUK infusions.

**Plasma and Urinary Parameters**

Table 3 shows plasma levels of electrolytes and creatinine. Plasma sodium and potassium were not influenced by RUK infusion or alacepril treatment. However, the plasma creatinine level was significantly decreased by RUK infusion and alacepril alone or in combination with RUK.

Table 4 shows alterations in urinary parameters. The high salt diet increased urinary volume and the urinary excretion of sodium; however, these parameters did not differ among the control and the three treatment groups.

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**TABLE 1. Systolic Arterial Pressure Measured by Direct Method**

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>Systolic Blood Pressure, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl-2% control (n=9)</td>
<td>222.2±5.4</td>
</tr>
<tr>
<td>Dahl-RUK (n=7)</td>
<td>219.4±6.7</td>
</tr>
<tr>
<td>Dahl-ALCP (n=11)</td>
<td>195.3±6.0*</td>
</tr>
<tr>
<td>Dahl-RUK+ALCP (n=9)</td>
<td>179.0±7.2*</td>
</tr>
<tr>
<td>Dahl-0.3% control (n=5)</td>
<td>132.2±2.2*</td>
</tr>
<tr>
<td>Pt&lt;.0001</td>
<td></td>
</tr>
</tbody>
</table>

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Dahl-2% control indicates Dahl salt-sensitive (Dahl S) rats fed a high salt diet; Dahl-RUK, Dahl S rats on a high salt diet given rat urinary kallikrein (RUK); Dahl-ALCP, alacepril-treated Dahl S rats on a high salt diet given RUK and alacepril; and Dahl-0.3% control (open circles with broken lines), low salt control Dahl S rats. Differences were assessed by two-way ANOVA. *P<.01, **P<.005, ***P<.0005 vs respective values in Dahl-2% control rats. TX indicates treatment.

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Dahl-2% control indicates Dahl salt-sensitive (Dahl S) rats fed a high salt diet; Dahl-RUK, Dahl S rats on a high salt diet given rat urinary kallikrein (RUK); Dahl-ALCP, alacepril-treated Dahl S rats on a high salt diet given both RUK and alacepril; and Dahl-0.3% control, control Dahl S rats fed a low salt diet.

*P<.005 vs Dahl-2% control.

†By one-way ANOVA. There was no difference in systolic blood pressure between Dahl-2% control and Dahl-RUK.
on a high salt diet. Urinary excretion of protein and NAG activity were significantly increased in control rats fed a high salt diet compared with normotensive Dahl S rats fed a low salt diet. The increased proteinuria was significantly attenuated by RUK infusion. Alacepril treatment did not decrease proteinuria, but the combination of RUK and alacepril significantly enhanced the excretion of vasodepressor PGF_2α. Alacepril treatment did not affect the excretion of 6-keto-PGF_1α and thromboxane, nor did the combined administration of RUK and alacepril.

Urinary Excretion of Bradykinin and cGMP

Table 6 shows urinary bradykinin excretion. RUK infusion produced a 145% increase in bradykinin excretion compared with excretion in the high salt control group. Treatment with alacepril alone did not increase bradykinin generation. However, the combination of alacepril with RUK increased bradykinin excretion by 164%.

Similarly, urinary cGMP excretion increased by 89% with RUK infusion, but the difference was not significant. However, the excretion was significantly increased by 123% with alacepril treatment, and it was further enhanced by 218% with the combined RUK and alacepril treatment.

Morphological Changes

Fig 5 shows the effects of RUK infusion on glomerular lesions. In hypertensive control Dahl S rats on a high salt diet, the number of glomeruli with a normal structure

### Table 1. Body and Organ Weights in Dahl Salt-Sensitive Rats

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>Body Weight, g</th>
<th>Heart Weight, g wet wt/100 g body wt</th>
<th>Weight of Aorta, mg wet wt/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl-2% control (n=9)</td>
<td>395±8</td>
<td>0.39±0.01</td>
<td>17.6±0.5</td>
</tr>
<tr>
<td>Dahl-RUK (n=7)</td>
<td>408±4</td>
<td>0.43±0.04</td>
<td>18.9±0.5</td>
</tr>
<tr>
<td>Dahl-ALCP (n=11)</td>
<td>408±6</td>
<td>0.37±0.01</td>
<td>16.9±0.3</td>
</tr>
<tr>
<td>Dahl-RUK+ALCP (n=9)</td>
<td>403±6</td>
<td>0.36±0.01</td>
<td>16.5±0.6</td>
</tr>
<tr>
<td>Dahl-0.3% control (n=5)</td>
<td>412±7</td>
<td>0.27±0.01</td>
<td>10.3±0.9</td>
</tr>
</tbody>
</table>

**P** NS <.05

**P** NS <.05

### Table 2. Alterations in Plasma Parameters

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>Na, mmol/L</th>
<th>K, mmol/L</th>
<th>Creatinine, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl-2% control (n=9)</td>
<td>157±2</td>
<td>4.8±0.1</td>
<td>0.066±0.005</td>
</tr>
<tr>
<td>Dahl-RUK (n=7)</td>
<td>158±1</td>
<td>4.9±1</td>
<td>0.047±0.004†</td>
</tr>
<tr>
<td>Dahl-ALCP (n=11)</td>
<td>157±2</td>
<td>4.9±0.1</td>
<td>0.050±0.005†</td>
</tr>
<tr>
<td>Dahl-RUK+ALCP (n=9)</td>
<td>156±2</td>
<td>4.8±0.2</td>
<td>0.047±0.003†</td>
</tr>
<tr>
<td>Dahl-0.3% control (n=5)</td>
<td>157±2</td>
<td>4.2±0.1</td>
<td>0.031±0.005†</td>
</tr>
</tbody>
</table>

**P** <.05, †P <.025 vs Dahl-2% control.

†By one-way ANOVA.
TABLE 5. Urinary Excretion of Eicosanoid

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>PGE(_1) nmol/d</th>
<th>6-keto-PGF(_{1α}) nmol/d</th>
<th>PGF(_{2α}) nmol/d</th>
<th>TXB(_\beta) nmol/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl-2% control (n=9)</td>
<td>0.550±0.046</td>
<td>0.465±0.065</td>
<td>0.378±0.028</td>
<td>0.074±0.008</td>
</tr>
<tr>
<td>Dahl-RUK (n=7)</td>
<td>0.512±0.051</td>
<td>0.334±0.045</td>
<td>0.400±0.044</td>
<td>0.065±0.006</td>
</tr>
<tr>
<td>Dahl-ALCP (n=11)</td>
<td>0.731±0.094</td>
<td>0.426±0.072</td>
<td>0.432±0.032</td>
<td>0.073±0.009</td>
</tr>
<tr>
<td>Dahl-RUK+ALCP (n=9)</td>
<td>0.760±0.056*</td>
<td>0.209±0.049</td>
<td>0.467±0.033</td>
<td>0.072±0.006</td>
</tr>
<tr>
<td>Dahl-0.3% control (n=5)</td>
<td>0.597±0.050</td>
<td>0.525±0.011</td>
<td>0.292±0.018</td>
<td>0.042±0.002*</td>
</tr>
</tbody>
</table>

P<.05  NS  NS  <.01

Rat groups are as defined in Table 1. PG indicates prostaglandin; TX, thromboxane.
*P<.02 vs Dahl-2% control.
†By one-way ANOVA.

(group 0) was decreased, and the number of glomeruli with more severe glomerular lesions (groups II, III, and IV) was increased. RUK infusion decreased the number of glomeruli with more severe lesions (groups II, III, and IV) and increased the number of glomeruli having a normal appearance (group 0). Alacepril alone also attenuated the glomerular lesions. The combination of alacepril with RUK produced a resolution of glomerular sclerosis, with the distribution pattern becoming similar to that in normotensive control rats. These changes were scored semiquantitatively (Fig 6, top). The overall glomerular sclerosis was decreased significantly with RUK infusion, approaching the level seen with alacepril alone or the combination of alacepril and RUK.

Fig 7 shows the distribution of arteries by various degrees of injury. Arteries from control rats on a low salt diet had a normal appearance (group 0) or only medial lesions (group I). The high salt diet decreased the percentage of normal arteries and increased the percentage of arteries with more severe injury (groups II or III). In contrast to its effect on glomerular lesions, RUK infusion did not attenuate arterial lesions in Dahl S rats, whereas alacepril alone or in combination with RUK greatly increased the number of arteries having a normal appearance (group 0). These alterations were also reflected in the overall score for arterial injury (Fig 6, middle). RUK infusion did not decrease the score, although alacepril alone or in combination with RUK significantly reduced the score to the level seen in normotensive control rats.

TABLE 6. Urinary Excretion of Bradykinin and Cyclic GMP

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>Bradykinin, nmol/d</th>
<th>Cyclic GMP, nmol/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl-2% control (n=9)</td>
<td>0.330±0.044</td>
<td>9.42±2.54</td>
</tr>
<tr>
<td>Dahl-RUK (n=7)</td>
<td>0.809±0.133*</td>
<td>17.80±4.45</td>
</tr>
<tr>
<td>Dahl-ALCP (n=11)</td>
<td>0.239±0.031</td>
<td>21.02±3.56*</td>
</tr>
<tr>
<td>Dahl-RUK+ALCP (n=9)</td>
<td>0.873±0.053†</td>
<td>30.03±4.81*</td>
</tr>
<tr>
<td>Dahl-0.3% control (n=5)</td>
<td>0.081±0.023†</td>
<td>4.17±0.39</td>
</tr>
</tbody>
</table>

P<.001  <.01

Rat groups are as defined in Table 1.
*P<.01, †P<.001 vs Dahl-2% control.
†By one-way ANOVA.
Fig 6. Bar graphs show overall injury scores calculated from weight values and percentages of each severity of injury. Top, Overall glomerulosclerotic (GS) score; middle, overall score of arterial injury; bottom, overall tubular injury in medullary rays. Rat groups are as defined in Fig 4 legend. Values were compared with respective values in control Dahl S rats fed a high salt diet.

Fig 6, bottom, shows the effect of RUK infusion on tubular injury. A high salt diet caused significant tubular injury in Dahl S rats compared with normotensive control rats. Both the RUK infusion and alacepril alone significantly attenuated the tubular injury; the combination of RUK with the ACE inhibitor further reduced the tubular injury.

Discussion

The enzymatic activity of kallikrein is species specific. To investigate the effects of kallikrein replacement on kidney function in rats, we purified rat tissue kallikrein (RUK) from pooled urine. The prepared RUK showed a single band on both native PAGE and SDS-PAGE, thereby confirming the RUK purity. We found that a bolus infusion of 100 ng or more of RUK into the jugular vein produced a transient (1- to 2-minute) fall in arterial blood pressure, which quickly returned to baseline. In the present study, we administered 700 ng RUK per 24 hours by continuous infusion, well within the subpressor dose.

Systolic blood pressure was time-dependently increased by a high salt diet. RUK infusion alone did not affect this blood pressure elevation. However, RUK significantly enhanced the antihypertensive action of the ACE inhibitor alacepril, suggesting that kininase II inhibition potentiates the effects of RUK on blood pressure.

Intriguingly, despite the failure of RUK alone to attenuate blood pressure elevation induced by a high salt diet, RUK significantly attenuated renal glomerular and tubular lesions. Morphological improvement also was reflected in a reduced proteinuria and increased glomerular filtration rate. These data strongly suggest that the attenuation of renal injury in Dahl S rats by RUK alone is mediated not by hemodynamic changes but by events following the RUK infusion. Improvement of arterial injury was not marked in rats given RUK alone; however, the improvement of arterial lesions by the combination of RUK with the ACE inhibitor was greater than that achieved by alacepril alone, suggesting that in addition to a greater hemodynamic effect of the RUK and alacepril combination, the arterial lesions may also be influenced directly by the kallikrein-kinin system.

Kallikrein initiates the cascade reaction in the kallikrein-kinin-prostaglandin system. Bradykinin stimulates prostaglandin synthesis and nitric oxide formation. These vasoactive substances may attenuate target-organ injury by directly inhibiting proliferation of endothelial
and smooth muscle cells and by balancing the platelet-vascular wall interaction.5-6 In the present study, we clearly demonstrated that urinary excretion of bradykinin increased twofold to threefold when RUK was infused alone or administered in combination with alacepril. Urinary excretion of PGE2 was not significantly changed when RUK was infused alone; however, the combination of RUK with alacepril significantly increased PGE2 generation. Similarly, cGMP excretion was increased by RUK infusion alone. The excretion became greater when alacepril was coadministered with RUK. cGMP is known to be influenced by atrial natriuretic peptides. Since cardiac mass was decreased by the treatment, the increase in cGMP formation might primarily reflect the metabolism of nitric oxide formation. These data suggest that activation of the kallikrein-kinin-prostaglandin–nitric oxide cascade is at least in part responsible for the observed attenuation of the renal injury produced by RUK infusion. To define the role of bradykinin in the action of RUK, it would be of interest to test whether these beneficial effects are abolished with the bradykinin type-2 receptor antagonist Hoe 140. These experiments are ongoing.

Recent studies have expanded our knowledge of the physiological roles of tissue kallikrein. Kizuki et al6 and Kitagawa et al28 recently found that kallikrein can hydrolyze some peptides other than kinogen, which has been believed to be a sole substrate of the enzyme, thereby exerting an effect on central nervous system function. Tissue kallikrein produces peptides from serum, which in turn stimulate biosynthesis of prostacyclin. The wider range of actions seems helpful in explaining the action of RUK, although further experiments are necessary.

Hydrostatic or shear stress on the vascular wall is a major cause of target-organ injury in hypertension. A reduction in blood pressure is often associated with a resolution of renal damage. However, it has been postulated that part of the organ injury is produced by mechanisms unrelated to the blood pressure elevation. Adequate blood pressure control does not necessarily resolve all target-organ injury.7,8 The metabolism of vasoactive substances generated regionally in the vascular wall and the kidney is important in producing a regression of organ damage.

In the present study, the ACE inhibitor did not decrease urinary protein excretion or urinary albumin excretion, whereas it significantly attenuated glomerular sclerotic lesions in Dahl S rats. There is much evidence that ACE inhibitors decrease urinary protein excretion particularly in diabetic nephropathy. The reason the ACE inhibitor failed to decrease proteinuria in the present study is not certain; however, it has been reported that a higher dose of captopril may increase urinary protein excretion, probably through a mechanism involving sulfhydryl moiety in the chemical structure.31,32 In addition, we have found that functional improvement by antihypertensive drug treatment may be preceded by an attenuation of glomerular sclerotic lesions.33 These various mechanisms appeared to underlie the unsatisfactory effect of alacepril on urinary protein excretion.

There is some evidence that kallikrein activity is lower in subjects with a family history of essential hypertension and that there is cosegregation of blood pressure with kallikrein gene restriction fragment length polymorphism.34-35 There is evidence that Dahl S rats exhibit an impaired kallikrein-kinin-prostaglandin axis even in the prehypertensive stage and that the hypertension in Dahl S rats is often associated with more severe target-organ injury. These data suggest that the kallikrein gene is very close to or is even a member of the genes responsible for the expression of events following hypertension.

In the present study, we demonstrated that kallikrein replacement increased activity of the kallikrein-kinin-prostaglandin axis and attenuated renal injury in hypertension. This may indicate that the replacement of RUK provides a useful additive in achieving the ultimate goal of antihypertensive treatment. Although we have no direct evidence supporting this hypothesis, clinical studies to test it would seem to be worthwhile. If the hypothesis is true, replacement of kallikrein using genetic techniques may prevent target-organ injury in essential hypertension. Additional studies are warranted to explore this intriguing possibility.

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Long-term infusion of kallikrein attenuates renal injury in Dahl salt-sensitive rats.

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