Sodium transport antagonism reduces thrombotic microangiopathy in stroke-prone spontaneously hypertensive rats

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MATERIALS AND METHODS

Experimental animals. Experiments were performed using 81 male SHRSP (generations F-82 to F-83) from our colony at New York Medical College. These animals were bred from National Institutes of Health stock, which was derived from the SHRSP/A3N substrain described originally by Okamoto and co-workers (27). Animals were weaned at 4 wk of age and then fed a standard rodent diet (Purina Lab Chow 5001; Ralston-Purina, St. Louis, MO) and allowed tap water ad libitum unless otherwise indicated by the experimental protocols. The rats were housed in a room at an ambient temperature of 22 ± 1°C with a 12:12-h light-dark cycle. All experiments were performed in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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accompanying the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86–23), and the Institutional Animal Care and Use Committee at New York Medical College approved all procedures. Protocol 1 (age-matched study). Twelve male SHRSP were placed on the Stroke-Prone Rodent Diet (39–288, Zeigler Bros., Gardeners, PA) and given 1% NaCl to drink starting at 58 days of age. The aforementioned diet has been reported to cause a higher incidence of stroke in SHRSP (44) and possesses a lower content of potassium (0.7 vs. 1.2% by weight) and protein (17 vs. 22% by weight) than the standard diet (37). All animals were housed individually and received metabolic cages for quantitative collection of urine on consecutive days. At 61 days of age, littersmates were separated into two groups: a control group (n = 6) that received 3 mg·kg⁻¹·day⁻¹ of deionized water (vehicle) and a 1.0 mg·kg⁻¹·day⁻¹ amiloride-treated group (n = 6). Treatments were administered by oral gavage using a soft rubber nelaton infant feeding tube (Rusch) and were divided between a morning dose and an evening dose each day. The amiloride dose was chosen based on data from our previous study showing that this dose resulted in a diuretic effect and a slight decrease in blood pressure. SBP of awake rats was measured at weekly intervals by tail-cuff plethysmography. After a 5-min stabilization period, an average of nine blood pressure readings was obtained from each rat. Arterial blood pressure was also measured from an in-dwelling femoral arterial catheter (protocol 1) in rats that were anesthetized with pentobarbital sodium (50 mg/kg ip; Abbott Laboratories). The trachea was cannulated with PE-240 tubing, and the rats were allowed to respire freely. Body temperature was maintained at 37°C using a thermostatically regulated heat lamp. The femoral artery was cannulated with PE-50 tubing containing heparinized saline (30 IU/ml) for arterial blood pressure measurement using a COBE CDX III fixed-dome transducer connected to a Digi-Med blood pressure analyzer (Micro-Med; Lexington, KY), which in turn was connected to a DPU-411 thermal printer.

Analytic procedures. PRA was measured byRIA of the ANG I generated during incubation of 1-ml aliquots of plasma with reagents provided in the assay kit (New England Nuclear, Boston, MA). PRA was expressed as nanograms of ANG I generated per millilitre of plasma per hour of incubation. BUN in serum samples was measured with a colorimetric diagnostic kit (Sigma). Urinary protein was measured by the sulfosalicylic acid turbidity method as previously described (37). Urinary sodium and potassium concentrations were measured using flame photometry. On the last day of treatment, at 96 days of age, rats were anesthetized and a femoral artery was cannulated for blood pressure measurement. After a 30-min period of blood pressure monitoring, 2 ml of blood were collected from the femoral artery into a chilled ETDA tube for the measurement of plasma renin activity (PRA). A serum sample was then obtained and used for the measurement of blood urea nitrogen (BUN). Kidneys were then excised and preserved in 10% neutral-buffered formalin for later histological examination. Protocol 2 (survival study). In a second protocol, male SHRSP littersmates were separated into three groups and given 1% NaCl to drink and fed the Stroke-Prone Rodent Diet starting at 8 wk of age. Animals were infused with propanediol (vehicle, 12 μl/day, n = 7), 5·(N,N-dimethyl-amiloride hydrochloride (A-4562; dimethylamiloride; 0.7 mg·kg⁻¹·day⁻¹, n = 8), or N-benzylmimidino-3,5-diamino-6-chloro-pyrazine-carboxamine hydrochloride) was purchased from Sigma (St. Louis, MO). Systolic blood pressure (SBP) was measured by tail-cuff plethysmography. Body weight and urine volume were measured each day. Urinary protein concentration was measured by the turbidity method, and urinary sodium and potassium concentrations were measured by flame photometry. On the last day of treatment, at 96 days of age, rats were anesthetized and a femoral artery was cannulated for blood pressure measurement. After a 30-min period of blood pressure monitoring, 2 ml of blood were collected from the femoral artery into a chilled ETDA tube for the measurement of plasma renin activity (PRA). A serum sample was then obtained and used for the measurement of blood urea nitrogen (BUN). Kidneys were then excised and preserved in 10% neutral-buffered formalin for later histological examination. Protocol 3 (plasma potassium study). In a third experimental series, 46 male SHRSP were fed the Stroke-Prone Rodent Diet (39–288, Zeigler Bros.) and given 1% NaCl solution to drink ad libitum starting at 12 wk and 3 days of age. Four days later, littersmates were divided into three treatment groups: one that received vehicle (1.0 ml/kg of propanediol, n = 15), one that received benzamil (1.0 mg/kg, n = 16), and one that received dimethylamiloride (1.0 mg/kg, n = 15). Treatments were administered as a single subcutaneous injection. At either 4 or 24 h after treatment, animals were anesthetized with pentobarbital sodium (50 mg/kg ip; Abbott Laboratories, North Chicago, IL) and, immediately on sedation, blood was obtained from the abdominal aorta through a midline incision. To minimize hemolysis, which may falsely elevate the level of plasma potassium, blood was drawn using large-bore (18-gauge) heparin-coated needles connected to heparin-coated 10-ml syringes. The blood was immediately centrifuged for 10-min at 3,000 rpm, and the plasma was separated and stored for later determination of sodium and potassium concentrations.

Experimental procedures. SBP of awake rats was measured using a Natsume KN 210 manometer and tachometer (Peninsula Laboratories, Belmont, CA). Rats were warmed at 37°C for 10 min and allowed to rest quietly in a Lucite chamber before tail-cuff plethysmography. After a 5-min stabilization period, an average of nine blood pressure readings was obtained from each rat. Arterial blood pressure was also measured from an in-dwelling femoral arterial catheter (protocol 1) in rats that were anesthetized with pentobarbital sodium (50 mg/kg ip; Abbott Laboratories). The trachea was cannulated with PE-240 tubing, and the rats were allowed to respire freely. Body temperature was maintained at 37°C using a thermostatically regulated heat lamp. The femoral artery was cannulated with PE-50 tubing containing heparinized saline (30 IU/ml) for arterial blood pressure measurement using a COBE CDX III fixed-dome transducer connected to a Digi-Med blood pressure analyzer (Micro-Med; Lexington, KY), which in turn was connected to a DPU-411 thermal printer.

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Histological evaluation. Coronal sections through the midportion of the kidneys were prepared for examination by light microscopy. Sections were cut at 3 μm and stained with hematoxylin and eosin and periodic acid-Schiff reagent. Renal damage was scored on a scale of 0 to 4 based on the severity and prevalence of damage as previously described (39). Glomerular injury was characterized by ischemic or thrombotic changes, whereas microvessels showed proliferative vasculopathy and/or fibrinoid necrosis with extravasation of fragmented erythrocytes with or without thrombosis. A score of 4 indicated extensive lesions affecting >20% of glomeruli and vessels; 3 indicated 15–20% involvement of moderate degree; 2 was assigned with 10–15% involvement with mild to moderate lesions; and 1 was assigned with <10% involvement of mild degree. A score of zero meant no overt morphological damage. The percentage of ischemic tubules was also evaluated. Statistical analysis. Significant effects with respect to treatment and time were determined by a two-way analysis of variance. Data with only one grouping variable were analyzed by one-way analysis of variance, followed by post hoc analysis using the method of Bonferroni. Ordinal data (histopathology scores) were analyzed using the Mann-Whitney independent rank sum test. Data were analyzed using the BMDP software package (BMDP Statistical Software, Los Angeles, CA). Differences between means were considered statistically significant at P < 0.05. Data are expressed as means ± SE. RESULTS Age-matched study. Vehicle-treated SHRSP exhibited a pronounced elevation in urinary protein excretion (107 ± 19 mg/day) at the end of the 5-wk treatment period (Fig. 1A). In contrast, urinary protein excretion remained at baseline levels (10–20 mg/day) throughout the study in SHRSP that were chronically treated with amiloride. Animals in both groups experienced a rise in SBP and remained hypertensive through-
out the study (Fig. 1B). However, there was no difference in SBP between the vehicle- and amiloride-treated groups. Also, there was no difference in SBP measured from a femoral arterial catheter in vehicle (233 ± 14 mmHg) vs. amiloride-treated SHRSP (240 ± 5 mmHg) at the end of the study (Table 1). Similarly, there was no difference in mean or diastolic arterial pressure (data not shown), and heart rate also did not differ among the groups (Table 1). All of the animals in the amiloride-treated group showed weight gain throughout the study; however, two animals in the vehicle-treated group began to lose body weight at 28 days of treatment (Fig. 2A). There were no differences in body weight or body weight gain among the groups over the treatment period. Urinary sodium excretion (U Na V) increased markedly on placement of the animals on a 1% NaCl drinking solution at 58 days of age (Fig. 2B). However, amiloride treatment had no effect on U Na V during the 5-wk period of treatment. The average daily U Na V during treatment was 11.9 ± 1.2 meq/day in the vehicle-treated group and 10.7 ± 1.0 meq/day in the amiloride-treated group (Table 1). Similarly, chronic treatment with amiloride did not affect average daily urinary volume or potassium excretion (U K V; Table 1). Amiloride prevented the marked increase in BUN and potassium excretion (U K V; Table 1). Amiloride prevented the marked increase in BUN and potassium excretion (U K V; Table 1).

Table 1. Average daily urinary excretions and terminal measurements in stroke-prone spontaneously hypertensive rats chronically treated with vehicle or amiloride

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (n = 6)</th>
<th>Amiloride (n = 6)</th>
</tr>
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<tr>
<td>UV, ml/day</td>
<td>35.6 ± 2.1</td>
<td>35.9 ± 2.1</td>
</tr>
<tr>
<td>U Na V, meq/day</td>
<td>12.3 ± 0.4</td>
<td>11.2 ± 0.5</td>
</tr>
<tr>
<td>U K V, meq/day</td>
<td>2.1 ± 0.1</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>233 ± 14</td>
<td>240 ± 5</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>388 ± 36</td>
<td>395 ± 18</td>
</tr>
<tr>
<td>BUN, mg%</td>
<td>27 ± 4</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Hematuria, vol%</td>
<td>37.1 ± 3.6</td>
<td>49.6 ± 1.3</td>
</tr>
<tr>
<td>PRA, ng·ml⁻¹·h⁻¹</td>
<td>17 ± 5</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>TMA score, 0–4</td>
<td>2.3 ± 0.4</td>
<td>0.1 ± 0.1†</td>
</tr>
<tr>
<td>Ischemic tubules, %</td>
<td>42.5 ± 9.3</td>
<td>0.8 ± 0.8‡</td>
</tr>
<tr>
<td>Age at death, days</td>
<td>96 ± 1</td>
<td>96 ± 1</td>
</tr>
</tbody>
</table>

Values are means ± SE. Male stroke prone spontaneously hypertensive rats (SHRSP) were maintained on 1% NaCl drinking solution and Stroke-Prone Rodent Diet starting at 58 days of age and subsequently treated with amiloride (1.0 mg·kg⁻¹·day⁻¹ by oral gavage) or vehicle (3 ml·kg⁻¹·day⁻¹ of deionized water) for 5 wk starting at 61 days of age. n, No. of rats; BP, blood pressure; U Na V, urinary sodium excretion; U K V, urinary potassium excretion; UV, urinary volume; BUN, blood urea nitrogen; PRA, plasma renin activity; TMA, thrombotic microangiopathy. *P < 0.05, †P < 0.01 vs. vehicle.

Fig. 1. Urinary protein excretion (UPE; A) and systolic blood pressure (SBP; B) of stroke-prone spontaneously hypertensive rats (SHRSP) chronically treated with either vehicle (n = 6) or amiloride (1.0 mg·kg⁻¹·day⁻¹, n = 6) starting at 61 days of age. All animals were fed the Stroke-Prone Rodent Diet to eat ad libitum starting at 58 days of age. Values are means ± SE. Chronic treatment with amiloride prevented the development of proteinuria compared with the vehicle-treated group. However, there was no difference in SBP between the groups. **P < 0.01 vehicle vs. amiloride.

Fig. 2. Body weight (A) and urinary sodium excretion (U Na V; B) of SHRSP chronically treated with either vehicle (n = 6) or amiloride (1.0 mg·kg⁻¹·day⁻¹, n = 6). All animals were given 1% NaCl to drink and Stroke-Prone Rodent Diet to eat ad libitum starting at 58 days of age. Values are means ± SE. Chronic treatment with amiloride did not affect body weight or U Na V over the 5-wk period of study.
and lowering of arterial hematocrit observed in vehicle-treated SHRSP at the end of 5 wk of treatment; however, PRA did not differ between the groups (Table 1).

The results for the renal histological analysis are presented in Table 1. Microvascular and glomerular lesions of thrombotic microangiopathy were significantly reduced in SHRSP chronically treated with amiloride (P < 0.01 compared with the vehicle-treated group). Figure 3 shows the histological appearance of the renal cortex from the animals in these groups at autopsy. Glomeruli, arteries and arterioles, tubules, and interstitium were essentially unremarkable in amiloride-treated SHRSP except for an occasional lesion of thrombotic microangiopathy. All vehicle-treated SHRSP revealed extensive renal microvascular and glomerular injury, as illustrated in Fig. 3B. These lesions are similar to those seen in human malignant nephrosclerosis. In addition to focally obliterative and massive fibrinoid necrosis of vessel walls, extravasation of fragmented erythrocytes in the vessel wall was quite prominent. A few glomeruli revealed ischemic retraction of capillary tufts, possibly secondary to arteriolar obliteration. The surrounding tubules frequently showed ischemic retraction. Scattered mononuclear leukocyte infiltration was seen in the adjacent interstitium in areas of glomerular and microvascular injury.

Survival study. Figure 4A shows the cumulative percent survival of saline-drinking SHRSP that were treated chronically with either vehicle, dimethylamiloride, or benzamil starting at 8 wk and 2 days of age. Five of seven vehicle-treated SHRSP showed signs of stroke, and all were dead at 12.7 ± 0.2 wk of age. In contrast, dimethylamiloride-treated SHRSP survived until 14.8 ± 0.6 wk of age (P < 0.005 vs. vehicle), and benzamil-treated SHRSP lived even longer (average age at death = 16.1 ± 0.2 wk; P < 0.0001 vs. vehicle and P < 0.05 vs. dimethylamiloride). SBP was 228 ± 5, 231 ± 4, and 226 ± 3 mmHg in vehicle-, dimethylamiloride-, and benzamil-treated SHRSP, respectively, at 10 wk of age and remained severely elevated with no differences among the groups throughout the study (Fig. 4B). Similarly, there was no difference in heart rate among the groups over the course of the study (data not shown). At 11 wk of age, urinary protein excretion in dimethylamiloride-treated (14 ± 1 mg/day) and benzamil-treated (14 ± 2 mg/day) SHRSP was significantly lower (P < 0.002) than in control SHRSP (41 ± 13 mg/day), which is consistent with a delayed onset of kidney damage (Fig. 5A). Similarly, the onset of proteinuria in the benzamil-treated SHRSP was delayed compared with dimethylamiloride-treated SHRSP. There was no difference in body weight among the groups until 11 wk of age (Fig. 5B). Thereafter, and consistent with our previous observations (34), vehicle-treated SHRSP began to show stroke signs, became debilitated, and lost body weight.

Plasma potassium study. Table 2 shows the results for plasma sodium, plasma potassium, and body weight at 4 and 24 h after the acute subcutaneous injection of vehicle, benzamil, or dimethylamiloride. There were no significant differences among the groups in plasma sodium concentration or body weight at either 4 or 24 h after treatment. However, there was a slight but significant elevation in plasma potassium concentration in benzamil- and dimethylamiloride-treated SHRSP compared with vehicle-treated control SHRSP at 4-h postdosing. There was no difference in plasma potassium among the groups at 24 h after administration of the treatments.

Fig. 3. Representative photomicrographs (hematoxylin and eosin; original magnification ×100) of renal cortex from saline-drinking, 96-day-old male SHRSP (A) showing severe lesions of thrombotic microangiopathy, characteristically seen in human malignant hypertension, at the end of the 5-wk period of treatment with vehicle. A small glomerulus reveals marked ischemic retraction (arrow), and another large glomerulus (double arrow) shows segmentally occlusive thrombosis. An adjacent small artery and arterioles are obliterated with nodular concentric myointimal proliferation and mural fibrinoid necrosis with extravasated and fragmented erythrocytes. Most of the tubules show ischemic change or simplified epithelium, whereas a few are dilated with proteinaceous casts indicative of proteinuria. Scattered mononuclear leukocytes are present in the surrounding interstitium. B: amiloride-treated SHRSP littermate shows essentially unremarkable renal histology at the end of the 5-wk period of treatment with 1.0 mg·kg⁻¹·day⁻¹. Note an interlobular artery (*) and an arteriole (arrow) with no significant pathology. C and D: higher magnifications of the representative fields from vehicle-treated and amiloride-treated SHRSP, respectively (hematoxylin and eosin; ×350).
DISCUSSION

In the present study, we observed a pronounced renal protective effect of amiloride on its daily administration for 5 wk in saline-drinking SHRSP. In agreement with the protective action previously observed against the occurrence of stroke (34), amiloride prevented the development of proteinuria, tubular ischemia, and severe renal vascular and glomerular lesions of thrombotic microangiopathy observed in vehicle-treated littermates at 96 days of age. Amiloride, administered at the same dose used in the present study, has also been reported to reduce glomerular scarring in a rodent model of high-salt/adriamycin-induced nephropathy (24). The beneficial effect observed in our study may have been due to the anesthesia and/or the microvascular damage in the vehicle-treated animals. These findings suggest the involvement of a sodium transport mechanism, presumably ENaC, in the development of thrombotic microangiopathy in saline-drinking SHRSP, which is unrelated

termination of the study. These data are consistent with the notion that elevated blood pressure, although necessary, is not solely responsible for vascular lesion development in these animals. The observation that BUN did not increase also supports the marked renal protective effect of amiloride. Similarly, a fall in hematocrit, the likely consequence of vascular injury and ensuing microangiopathic hemolytic anemia and red blood cell extravasation from vessels (as seen in Fig. 3A) and lowered erythropoietin levels as a result of renal parenchymal damage, was also prevented by amiloride. The protective effect of amiloride could not be accounted for by a decrease in PRA, as there was no significant difference among the groups. Amiloride has been reported to increase PRA in hypertensive patients (29); however, the inability to observe this effect in our study may have been due to the anesthesia and/or the microvascular damage in the vehicle-treated animals. These findings suggest the involvement of a sodium transport mechanism, presumably ENaC, in the development of thrombotic microangiopathy in saline-drinking SHRSP, which is unrelated
SHRSP rats were chronically treated with 50 mg/kg of dimethylamiloride would be consistent with inhibition of ENaC and NHE. To evaluate which transport mechanism may provide the greatest increase in survival, we performed a second experimental series to examine whether compounds having varying degrees of selectivity for inhibition of ENaC and NHE would mimic the effects of amiloride on survival in SHRSP. Benzamil has been reported to inhibit ENaC at nanomolar concentrations with little or no NHE-inhibitory activity (7, 14, 20). In contrast, dimethylamiloride was less effective than benzamil in increasing survival but was protective nonetheless. Because the primary end point in this study was mortality, a histological analysis was not performed, as all animals were injected subcutaneously with either 1.0 mg/kg of benzamil, or 1.0 mg/kg of dimethylamiloride. Animals were anesthetized with pentobarbital sodium (50 mg/kg, ip), and at either 4 or 24 h after the injection, blood was removed from the abdominal aorta into heparin-coated syringes for analysis of plasma electrolyte levels. *P < 0.05 vs. vehicle-treated SHRSP by 1-way analysis of variance.

Values are means ± SE. All animals were given 1% NaCl in the drinking water and Stroke-Prone Rodent Diet to eat ad libitum starting at 12.5 wk of age. Four days later, animals were injected subcutaneously with either 1.0 mg/kg of benzamil, or 1.0 mg/kg of dimethylamiloride. Animals were anesthetized with pentobarbital sodium (50 mg/kg, ip), and at either 4 or 24 h after the injection, blood was removed from the abdominal aorta into heparin-coated syringes for analysis of plasma electrolyte levels. *P < 0.05 vs. vehicle-treated SHRSP by 1-way analysis of variance.

to changes in arterial pressure, PRA, or day-to-day urinary water and electrolyte excretion.

Amiloride has been reported to act through inhibition of ENaC and NHE. To evaluate which transport mechanism may play a more significant role, we performed a second experimental series to examine whether compounds having varying degrees of selectivity for inhibition of ENaC and NHE would mimic the effects of amiloride on survival in SHRSP. Benzamil has been reported to inhibit ENaC at nanomolar concentrations with little or no NHE-inhibitory activity (7, 14, 20). In contrast, dimethylamiloride has been used extensively as an NHE inhibitor. Thus, we performed experiments to compare benzamil and dimethylamiloride for protection against stroke in saline-drinking SHRSP. The vehicle-treated SHRSP died sooner than in protocol 1, and this underscores the need for the inclusion of littermate controls in each experimental series. Other possible explanations include differences in the vehicle employed and/or the use of osmotic minipumps and the surgery to implant them. However, consistent with the notion that thrombotic microangiopathy is mediated by an ENaC mechanism, benzamil mimicked the effect of amiloride and markedly prolonged survival. In contrast, dimethylamiloride was less effective than benzamil in increasing survival but was protective nonetheless. Because the primary end point in this study was mortality, a histological analysis was not performed, as all animals had been found dead and the groups could not be matched for age. The increase in survival of animals treated with dimethylamiloride would be consistent with inhibition of ENaC and NHE mechanism; however, in an unpublished study we found no effect on the survival or proteinuria in saline-drinking SHRSP that were chronically treated with 50 mg·kg⁻¹·day⁻¹ of cariporide, a selective inhibitor of the NHE-1 subtype (12). Interestingly, in the two plasma samples that we submitted for HPLC analysis (National Medical Services, Willow Grove, PA) significant levels of amiloride (4.5 and 6.9 ng/ml) were detected in dimethylamiloride-treated SHRSP at 4 h postdosing. The presence of amiloride is consistent with the observation that there was a slight but significant increase in plasma potassium 4 h postdimethylamiloride. These data suggest that when dimethylamiloride is administered in vivo, it may be converted to amiloride, which, in turn, may be responsible for the delay in the onset of stroke via inhibition of ENaC.

In a separate experimental series, plasma levels of potassium were measured at 4 and 24 h after administration of benzamil or dimethylamiloride as an index of the renal action of these agents. The dose of benzamil and dimethylamiloride used was 50% greater than the total dose used in protocol 2 and was administered at once rather than infused slowly over a 24-h period to optimize the impact of these agents. Despite this, only a mild increase in plasma potassium was observed at 4 h, and no increase was observed at 24 h. Although an increase in plasma potassium with dimethylamiloride was unanticipated, this may be explained by our finding that amiloride was present in the bloodstream of these rats (see above). In a previous study, we found that bilateral adrenalectomy failed to produce hyperkalemia in saline-drinking SHRSP (plasma potassium concentration = 3.2 ± 0.3 meq/l in sham-operated SHRSP vs. 3.5 ± 0.3 meq/l in adrenalectomized SHRSP) but markedly decreased plasma aldosterone, prevented proteinuria, and abrogated thrombotic microangiopathy (9). We cannot be certain why saline-drinking SHRSP did not exhibit a hyperkalemic response to benzamil or adrenalectomy. This may relate to the reduced potassium content of the Stroke Prone Rodent Diet (37, 44). These animals were also maintained on a high-sodium intake, and sodium has been shown to interfere with the ability of amiloride to inhibit sodium conductance in vitro (3). Furthermore, amiloride has been reported to produce less potassium retention in SHR, the progenitor strain of SHRSP, than in normotensive Wistar rats (19). The absence of a hyperkalemic response is probably due to a combination of reduced potassium intake, increased sodium intake, and a genetic defect in potassium transport in the distal nephron of these rats. These observations suggest that renal protective effects observed in these animals cannot be explained by marked and persistent perturbations in plasma potassium levels, although we cannot exclude the possibility that some benefit may have been conveyed by minor changes in plasma potassium.

The mechanism by which amiloride and benzamil offer vascular protection in saline-drinking SHRSP remains unclear. Amiloride and benzamil are powerful direct inhibitors of ENaC, with other actions requiring higher concentrations. The possibility that inhibition of ENaC is responsible for the vascular protective effect is in keeping with our findings of vascular protection with the MR antagonists spironolactone and eplerenone (30, 31). Both of these agents interfere with ENaC function. MR blockade with canrenone (11) or spironolactone (1) did not inhibit aldosterone stimulation of NHE, which is generally held to be a nongenomic effect of this steroid hormone (43). However, the water soluble, open E-ring MR-antagonist RU-28318 did block the acute effects of aldosterone on NHE, which suggests that this nongenomic effect may be mediated by a mechanism involving the classic MR (1). Our results with amiloride, benzamil, and MR antagonists, taken together, provide strong evidence that blocking ENaC can prevent microvascular damage in SHRSP. A direct comparison of the vascular protective effects offered by the above classes of agents is complex, as hormones other than aldoste-

<table>
<thead>
<tr>
<th>Property</th>
<th>Vehicle</th>
<th>Benzamil</th>
<th>Dimethylamiloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium, meq/l</td>
<td>145.2±2.4</td>
<td>143.7±3.9</td>
<td>143.8±2.9</td>
</tr>
<tr>
<td>Potassium, meq/l</td>
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<td>3.3±0.2 *</td>
<td>3.4±0.4 *</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>271.9±8.6</td>
<td>271.3±7.5</td>
<td>273.4±4.5</td>
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<td>4 h postdosing</td>
<td>144.2±3.9</td>
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<td>24 h postdosing</td>
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<td>274.8±6.3</td>
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<td>267.5±6.2</td>
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<tr>
<td>No. of rats</td>
<td>7</td>
<td>8</td>
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Table 2. Plasma electrolyte levels and body weight in SHRSP rats receiving an acute injection of vehicle, benzamil, or dimethylamiloride.
ron, such as vasopressin (33), may also increase ENaC activity, and MR antagonism has been reported to substantially decrease the abundance of the thiazide-sensitive Na-Cl cotransporter (26). In addition to ENaC, amiloride is a potent inhibitor of other members of the degenerin/ENaC superfamily of ion channels (4). Thus other mechanisms by which amiloride may offer protection include inhibition of acid-sensing ion channels (40), mechanosensitive channels (8, 13), and nonselective cation channels (41). These actions of amiloride may reside at sites outside the distal tubular epithelium. Amiloride has been reported to abrogate >95% of the aldosterone-induced sodium influx into vascular smooth muscle cells after 2-wk exposure to aldosterone (21). MR-mediated signaling also regulates the ion-gated sodium channel in vascular endothelial cells, which requires an intact cytoskeleton (15). This site of action may be of particular significance to the effects observed in SHRSP, as endothelial injury is believed to initiate the development of thrombotic microangiopathy (32). In addition, experiments have shown the presence of an amiloride-sensitive channel on brain microvascular endothelial cells that is permeable to both sodium and potassium and may play an important role in the transfer of sodium and potassium across the blood-brain barrier (42). Central nervous system effects have also been proposed, as infusion of low doses of amiloride into the cerebral ventricles, but not when given in the peripheral circulation, can lower blood pressure in rats receiving a systemic infusion of deoxyxycorticosterone acetate (16, 17).

In conclusion, our findings demonstrate that chronic administration of amiloride produces marked protection against renal microvascular damage and proteinuria and increases in BUN in saline-drinking SHRSP that are matched for age. This protective effect could not be accounted for by differences in SBP, urinary water and electrolyte excretion, or PRA. Chronic treatment with the selective ENaC inhibitor benzamil and less so with the selective NHE inhibitor dimethylamiloride significantly prolonged survival of saline-drinking SHRSP as was previously reported for amiloride. The low doses of the inhibitors employed in this study were not associated with major or sustained alterations in SBP or plasma potassium. The pharmacological profile of protection obtained implicates a role for an ENaC-mediated sodium transport process in the evolution of stroke and renal vascular injury in saline-drinking SHRSP, although a contribution of NHE inhibition cannot be entirely excluded.

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REFERENCES


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