Mineralocorticoid Blockade Reduces Vascular Injury in Stroke-Prone Hypertensive Rats

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Abstract—Chronic treatment of saline-drinking stroke-prone spontaneously hypertensive rats (SHRSP) with agents that interfere with the formation or actions of angiotensin II (Ang II) prevents the development of stroke and renal vascular damage Ang II, in addition to its direct vascular effects, stimulates the synthesis and release of aldosterone To assess the role of aldosterone in the development of pathologic changes in these rats, we implanted time-release pellets containing 200 mg of the mineralocorticoid receptor antagonist, spironolactone, into 14 SHRSP at 7.5 weeks of age Eight SHRSP intemates received placebo pellets Over the period of study (3 to 4 weeks), systolic blood pressure (SBP) was not different between the groups Spironolactone did not enhance water and electrolyte excretion All placebo-treated SHRSP developed marked proteinuria (150±6 mg/d) whereas in spironolactone-treated SHRSP, urinary protein excretion (UPE) averaged 39±9 mg/d (P<0.001) In a second study to assess effects on survival, 6 SHRSP received spironolactone (10 mg/kg/d) and 6 received vehicle All but one of the control rats displayed signs of stroke and died by 16 weeks of age, while the spironolactone-treated SHRSP remained asymptomatic through 19 weeks of age (P<0.05) At 16 weeks of age, spironolactone-treated SHRSP were severely hypertensive (247±9 mm Hg), yet UPE remained at baseline levels In contrast, prettermal UPE averaged 136±13 mg/d in control rats (P<0.001) In both studies, histopathologic examination revealed a marked protective effect of spironolactone against the development of malignant nephrosclerotic and cerebrovascular lesions These observations indicate a vascular and end organ protective effect of spironolactone in the absence of lowered blood pressure in saline-drinking SHRSP and are consistent with a major role for mineralocorticoids as hormonal mediators of vascular injury (Hypertension. 1998;31[part 2]:451-458.)

Key Words: hypertension • kidney • malignant nephrosclerosis • spironolactone • stroke

In 1972, clinical studies by Brunner and coworkers identified plasma renin activity (PRA) and aldosterone as independent risk factors for heart attack and stroke They found that among the patients who developed strokes and myocardial infarctions, all had normal or high PRA, and aldosterone secretion Previous studies by our group and others have provided experimental evidence to support a role for the renin-angiotensin-aldosterone system (RAAS) in the development of vascular injury, as angiotensin converting enzyme (ACE) inhibitors and Ang II receptor antagonists prevented the development of stroke and malignant nephrosclerosis in SHRSP Since these studies were conducted in salt-loaded SHRSP, which respond to these agents with minimal blood pressure lowering, they provided evidence for a pathophysiologic role for Ang II in the development of vascular lesions of malignant nephrosclerosis independent of severely elevated blood pressure Consistent with a role for Ang II was the finding that SHRSP display a paradoxical increase in PRA with time, despite continued salt-loading 19,60 Although Ang II stimulates the synthesis and release of aldosterone,11,12 in addition to its direct vascular actions, a role for mineralocorticoids in the pathology of SHRSP has not previously been evaluated This possibility is supported by the finding that lesions of malignant nephrosclerosis and stroke have been classically described in rats with mineralocorticoid hypertension induced by the chronic administration of deoxycorticosterone acetate (DOCA) and salt 11-15 The renal lesions that develop in these animals are characterized by fibrinoid necrosis of vessels and proliferative arteriopathy Such lesions are identical to those that we12 and other investigators11,12 have observed in saline-drinking SHRSP and are also seen in Ang II-salt hypertensive rats 12,16 These observations have led us to hypothesize that mineralocorticoids play a role in the development of vascular injury in saline-drinking SHRSP To test this hypothesis we chronically treated SHRSP with spironolactone to determine whether mineralocorticoid receptor blockade would alter the development of pathology in these animals

Methods

Animals

Studies were conducted using male SHRSP/A3N (generations F-75 and P-77), n=34, from our local colony and were approved by the Institutional Animal Care and Use Committee All animals were housed in a room lighted 12 hours per day at an ambient temperature of 22±1°C in the Animal Care Facility at New York Medical
65 weeks of age Rats were handled and weighed daily. At 7.5 weeks of age, time-release pellets containing 200 mg of spironolactone were implanted in 8 heterotopic controls. Pellets were implanted subcutaneously at the nape of the neck through a skin incision in animals receiving inhalatory anesthesia with isoflurane. The average daily dose of spironolactone was calculated to be 17 mg/kg at the start of the study and 13 mg/kg at the end of the study, which was 3 to 4 weeks later. This dose of spironolactone is slightly higher than the dose of 10 mg/kg, which was previously used. Aldosterone to tissues in vivo is 5. After implantation, all animals were housed indvidually in metabolic cages at 10 weeks of age so that measurements of 24-hour food and saline intake and urine output were measured before and following surgery. Treatments were given Stroke-Prone Rodent Diet (#39-288, Zelgler Bros Inc) and 1% NaCl drinking solution ad libitum. Twenty-four hour fluid and food intake, and urine output were measured before and after surgery and each week thereafter. Urine was collected for the determination of protein and electrolyte excretion. SBP and heart rate were measured each week by tail-cuff plethysmography. Treatments were continued until 10.5 to 11.5 weeks of age. At that time, trunk blood was collected into chilled EDTA tubes after rapid decapitation of animals. The blood samples were centrifuged for 10 minutes at 4°C and 3000 rpm to obtain plasma, which was then stored below -20°C for later radiolmmunoassay for PRA. The brain, heart, kidneys, and adrenal glands were removed, blotted dry, and immediately weighed and fixed. Brains and kidneys were further processed for light microscopic evaluation.

**Statistical Analysis**

Significant effects with respect to treatment and time were determined by two-way analysis of variance. Data with only one group factor were analyzed statistically by one-way analysis of variance followed by post-hoc analysis using the method of Bonferroni. The Kaplan-Meier method was used for comparison of cumulative percent survival curves. Ordinal data (brain lesion scores) were analyzed using the Mann-Whitney nonparametric test. Data were analyzed using version 2 01 of the GraphPad Prism statistical software package (GraphPad Software Inc). A value of $P<0.05$ was considered to be statistically significant. Data are reported as mean±SE.
Protocol 1

SHRSP from both groups showed a progressive increase in SBP with age (Fig 1A) By the end of the study, animals in both groups were severely hypertensive No significant differences in SBP were observed between the groups Likewise, heart rate was unchanged by chronic treatment with spironolactone and showed little change over the course of the study (data not shown) There was no difference in UPE between the groups through 9 weeks of age (Fig 1B) Thereafter, UPE increased markedly in SHRSP receiving placebo and averaged 150±6 mg/d at 10 4 weeks of age In contrast, UPE remained at low levels and averaged 22±5 mg/d at 10 4 weeks of age in the spironolactone treated group (P<0001) All animals were sacrificed in the ensuing week Preterminal UPE averaged 39±9 mg/d in the spironolactone-treated animals Body weight (Fig 1C) increased progressively in both groups through 9 5 weeks of age at which time the weight of placebo-treated animals began to decline Fig 2 shows the results for urine volume, sodium excretion, and potassium excretion After placement of the animals on high salt/stroke prone diet, urine volume and sodium excretion increased markedly (P<0001) whereas potassium excretion remained unchanged The urinary Na⁺/K⁺ ratio increased from 0 51±0 02 to 5 97±0 24 (P<0001) Urine volume, sodium excretion, and the urinary Na⁺/K⁺ ratio were comparable in both the spironolactone- and placebo-treated groups until 10 3 weeks of age, at which time urine output, sodium excretion, and the urinary Na⁺/K⁺ ratio were greater in the placebo-treated group Urinary potassium excretion was not different between the groups during the study period There were no significant differences in heart weight (1 4±0 05 versus 1 52±0 06 g), total kidney weight (2 56±0 14 versus 2 55±0 06 g), total adrenal weight (64±2±5 versus 57 6±5 2 mg) and brain weight (2 05±0 05 versus 2 0±0 04 g) between placebo- and spironolactone-treated SHRSP, respectively

The Table summarizes the histopathologic findings in the brains and kidneys of SHRSP The average cerebrovascular lesion score in spironolactone-treated animals was significantly less than in placebo-treated SHRSP Microscopic examination revealed cerebrovascular lesions in the brains of all placebo-treated SHRSP Lesions included moderate to severe edema
and rarefaction, spongiform necrosis, and hemorrhagic lesions. Brains from spironolactone-treated animals showed occasional lesions that were not as marked as in the placebo-treated group. Representative photomicrographs of renal cortex from SHRSP given either a placebo pellet or a time-release pellet containing spironolactone are shown in Figs. 3A and 3B, respectively. Prominent glomerular and vascular lesions of thrombotic microangiopathy characteristic of malignant nephrosclerosis were noted in placebo-treated SHRSP. Vascular lesions were primarily confined to renal cortex and affected medium- to small-sized interlobular arteries as well as arterioles. Vascular lesions consisted predominantly of the thrombotic type. Proliferative lesions were noted in lesser numbers. The remaining vessels without lesions frequently showed medial thickening. Rarely, an arcuate size artery revealed focal fibrinoid necrosis. Glomeruli revealed predominantly ischemic retraction of capillary tufts with or without mesangiolysis. These were probably secondary to preglomerular vascular occlusion. A few additional glomeruli were markedly swollen and showed thrombotic lesions. In contrast to these changes in placebo-treated SHRSP, spironolactone-treated SHRSP exhibited a marked reduction in both renal vascular and glomerular lesions (Table). No appreciable leukocytic infiltrate was observed in the kidneys of either group.

PRA averaged 52±15 ng Ang I/mL/h in placebo-treated SHRSP, which is consistent with the paradoxic increases that are known to occur in salt-loaded SHRSP. PRA averaged 16±2 ng Ang I/mL/h in the spironolactone-treated SHRSP, which, although elevated, was significant less than in their placebo-treated littermates (P<0.01).

**Protocol 2**

In a separate experimental series, SHRSP were started on Stroke-Prone Rodent Diet and 1% NaCl drinking solution at 8-8 weeks of age and were chronically treated with either spironolactone (n=6) or vehicle (n=6). Fig 4A shows the cumulative percentage of spironolactone- and vehicle-treated animals surviving at different ages. Five out of the 6 SHRSP from the vehicle-treated group displayed signs of stroke beginning at 13.4 weeks of age and died before 16 weeks of age. In contrast, none of the spironolactone-treated SHRSP showed stroke signs and all survived until 19 weeks of age at which point the study was ended. Microscopic examination revealed cerebrovascular lesions in the brains of all vehicle-treated SHRSP, commensurate with the demonstration of stroke signs in these animals. Brains from spironolactone-treated animals showed occasional lesions of rarefaction and edema that were not as marked as in the controls. The average cerebrovascular lesion score in spironolactone-treated animals was significantly less than in the control group (0.8±0.6 versus 2.5±0.5, P<0.05). There was no significant difference in systolic blood pressure between the groups over the course of the study (Fig 4B). At 15 weeks of age, spironolactone-treated SHRSP developed severe hypertension, averaging levels of 259±5 mm Hg. Saline intake and urine volume tended to increase with time while food intake and body weight tended to decrease, however, there were no statistically significant differences between the groups (data not shown). Preterminal UPE in these animals reached 1.36±1.9 mg/d while levels in spironolactone-treated animals remained at basal levels (17±2 mg/d, P<0.01). The occurrence of renal vascular and glomerular lesions was also markedly reduced in the kidneys of those SHRSP that were chronically treated with spironolactone. The percentage of lesioned glomeruli was 21±2 in the vehicle group and 6±4 in the group treated with spironolactone, P<0.01. The number of vascular profiles with lesions per 100 glomeruli counted was 28±4 in the vehicle group and 6±4 in the group treated with spironolactone, P<0.01.

**Discussion**

To determine whether endogenous mineralocorticoids play a role in the malignant nephrosclerosis and stroke that develops in saline-drinking SHRSP, we chronically administered a mineralocorticoid receptor antagonist to these animals. We found that a low dose of spironolactone, 10 mg/kg/d, markedly increased the survival of saline-drinking SHRSP as compared to the vehicle-treated group. In this experiment, mortality from stroke in the vehicle group also occurred at a somewhat later age relative to that seen in our previous studies. This delay is best explained by the fact that these animals were started on the high-salt/stroke-prone rodent diet at a later age. In a separate experimental series, we started the animals on high-salt/stroke-prone rodent diet at the usual time. Survival was not an endpoint in this study, nonetheless, 3 of the placebo-treated rats developed neurological signs of stroke and died early. Histopathologic analysis of the brains from both of these experiments demonstrated a significant reduction in cerebrovascular damage in those SHRSP given spironolactone. Previous studies by Robert et al. demonstrated that rats given DOCA and a 1% NaCl drinking solution developed neurological signs of stroke and cerebrovascular lesions. Recent studies by McLeod et al. have demonstrated that chronic infusion of aldosterone can reverse the ability of captropl to prevent mortality and cerebrovascular injury in salt-loaded
Figure 3. Photomicrographs of representative hematoxylin and eosin-stained mid-coronal kidney sections from two 10.4 week-old stroke-prone spontaneously hypertensive rats maintained on Stroke-Prone Rodent Diet and 1% NaCl starting at 7.5 weeks of age (original magnification, x 130). (A) Renal cortex from an animal into which a placebo pellet was implanted at 7.5 weeks of age. The photomicrograph illustrates the presence of lesions of thrombotic microangiopathy affecting several glomeruli and blood vessels. A relatively normal glomerulus is seen towards the right lower quadrant. Ischemic retraction and obliteration of capillary tufts is seen in 1 glomerulus (small arrow). Segmental capillary tuft necrosis, widespread thrombosis, and cellular swelling, are evident in two additional swollen glomeruli (large arrows). Microvascular lesions consist of marked concentric medial hypertrophy (small arrowhead), and circumferential, transmural fibrinoid necrosis as observed in two small arteries (large arrowheads), one showing fragmented and extravasated erythrocytes, in addition. (B) Renal cortex from an animal into which a pellet containing 200 mg of spironolactone was implanted at 7.5 weeks of age. The photomicrograph illustrates the absence of significant glomerular or vascular pathology as seen above in the age-matched, placebo-treated littermate control.

The results of the present study are consistent with these findings and suggest that endogenous mineralocorticoids play a role in the development of cerebral lesions in saline-drinking SHRSP.

Spironolactone also produced a marked protective effect against the development of renal vascular injury in saline-drinking SHRSP. In both experimental protocols, animals treated with the mineralocorticoid receptor antagonist developed less proteinuria and exhibited substantial reductions in the number of glomerular and vascular lesions. In previous studies, we found that chronic treatment with ACE inhibitors and the Ang II type 1 receptor antagonist, losartan, agents that would be expected to diminish aldosterone release, prevented lesions of malignant nephrosclerosis despite the...
Mineralocorticoids in Stroke-Prone SHR

absence of a blood pressure lowering effect in saline-drinking SHRSP. Chronic ACE inhibitor treatment with enalapril also failed to lower arterial pressure in rats with DOCA-salt hypertension but, in contrast to SHRSP, did not protect against the development of proteinuria and malignant nephrosclerosis. Aldosterone has also been reported to play a role in the development of renal injury in the remnant kidney model of chronic renal failure. In that study, exogenous aldosterone administration completely reversed the ability of combined treatment with enalapril and losartan to attenuate proteinuria, hypertension, and glomerular sclerosis. Although the ultimate development of glomerular sclerosis was not prevented, chronic administration of a high dose of spironolactone significantly delayed the development of proteinuria in the remnant kidney model of renal failure. In a recent study we also found that aldosterone infusion can completely reverse the renal protective action of captopril in saline-drinking SHRSP. The findings in the present study with spironolactone provide strong evidence for a major role of endogenous mineralocorticoids in the development of renal vascular pathology of saline-drinking SHRSP. A relationship between mineralocorticoids and malignant nephrosclerosis in rats was first demonstrated by Selye and coworkers in 1943. They reported that combined treatment with DOCA and a 1% NaCl-drinking solution produced severe hypertension and malignant nephrosclerosis while DOCA was comparatively inactive when NaCl intake was not excessive. The contribution of mineralocorticoids to vascular injury may have been particularly prominent under the conditions of our study, since SHRSP were maintained on a 1% NaCl-drinking solution.

In the present study PRA was markedly elevated in placebo-treated SHRSP, which is consistent with the paradoxical increase known to occur with salt-loading in these animals. PRA averaged 16.0±2.0 ng Ang I/mL/h in spironolactone-treated SHRSP. Although this value was less than in placebo-treated SHRSP, it was substantially elevated compared to our previously reported values of 3.5±1.0 ng Ang I/mL/h in WKY given standard diet and water, 0.6±0.5 ng Ang I/mL/h in WKY given Stroke-Prone Rodent Diet and 1% NaCl, and 9.2±2.5 ng Ang I/mL/h in SHRSP given standard diet and water. The higher level of PRA in placebo-treated SHRSP relative to that of spironolactone-treated SHRSP is probably due to the extensive renal damage that was observed in the former group (Table). Consistent with the concept that the protective effect of spironolactone relates to mineralocorticoid receptor antagonism rather than effects on PRA is the finding that aldosterone can restore the development of cerebral vascular and renal vascular injury in salt-loaded SHRSP chronically treated with the ACE inhibitor captopril.

The protective effect of spironolactone treatment against end organ damage in SHRSP appears to be independent of blood pressure lowering, since limited, if any, reduction in blood pressure was observed in either of the study protocols. Although in some instances blood pressure tended to be lower in spironolactone-treated rats, this may reflect the decreased renal vascular damage in this group. These observations are similar to the finding that ACE inhibitors afford vascular protection in saline-drinking SHRSP with little or no reduction in systemic blood pressure. In salt-loaded rats receiving a peripheral infusion of aldosterone, concomitant intracerebroventricular infusion of a mineralocorticoid receptor antagonist, RU28318, abolished aldosterone-induced hypertension but did not affect the production of cardiac hypertrophy or fibrosis. These findings provide evidence for a central nervous system component to the hypertensive effect of aldosterone as previously described by Gomez-Sanchez and also support a dissociation between blood pressure and end organ damage.

The beneficial effects of spironolactone in saline-drinking SHRSP were also independent of major changes in water and electrolyte excretion. Chronic administration of mild diuretics is typically associated with only a transient increase in water and electrolyte excretion that is not sustained. In rats on a normal sodium intake, administration of spironolactone at a dose of 20 mg/kg/d for one week did not alter daily urinary potassium excretion. Increases in urinary sodium excretion (10%) and the urinary Na+/K+ ratio (15%) were observed only on the first day of treatment. We observed no differences in water and electrolyte excretion between the groups until the onset of renal damage, signified by proteinuria, at which time urine output and sodium excretion were higher in placebo-treated rats. Smeda and Tkachenko examined the effects of

Figure 4. Line graphs showing (A) cumulative percent survival and (B) systolic blood pressure (SBP) of stroke-prone spontaneously hypertensive rats that were chronically treated with either spironolactone (10 mg/kg/d SC, n=6) or the sesame oil vehicle (1 mL/kg SC, n=6). Animals were maintained on 1% NaCl-dinking solution and Stroke-Prone Rodent Diet starting at 8.8 weeks of age at which time treatments were started. Values in (B) are mean±SE. Number in parentheses indicates the number of animals.
various diuretics on survival of salt-loaded SHRSP. They found that chronic treatment with chlorothiazide or amiloride offered no protection against stroke and concluded that increases or decreases in urinary potassium excretion do not affect the development of pathology in these animals. Also in this study, furosemide treatment decreased survival of SHRSP, which was thought to be caused by activation of the RAAS. Previous studies have demonstrated a protective effect of high dietary potassium against the development of stroke in SHRSP, which was not associated with increases in plasma potassium levels. Thus, increases in serum potassium, per se, may not play a major role in the vascular protective effect of high dietary potassium in salt-loaded SHRSP. Studies by Volpe and coworkers demonstrated that the protective effect of high dietary potassium in salt-loaded SHRSP was most likely due to suppression of renn release and not diuresis and natriuresis. Likewise, we found that chronic treatment with enalapril or captopril had no effect on water and electrolyte excretion by saline-drinking SHRSP but prevented and organ damage. Our results with spironolactone are commensurate with these findings and support the concept that the protective effect with this treatment is not due to major changes in water and electrolyte excretion.

The precise mechanism by which mineralocorticoids contribute to the development of vascular pathology in saline-drinking SHRSP remains unclear. Chronic treatment with spironolactone has been reported to prevent myocardial fibrosis in rats with hypertension induced by unilateral renal ischemia, chronic aldosterone infusion and renal lesions of malignant nephrosclerosis and signs of vascular pathology, which was first proposed by Masson and colleagues. Thus, an interaction between Ang II and aldosterone and Ang II in vascular smooth muscle cells, thereby increasing the synthesis of montol-1,4,5-inphosphates, should be further investigated. Aldosterone and Ang II were found to increase protein kinase C activity in vascular smooth muscle cells and protein kinase C activation has been reported to increase vascular permeability. Ulhan and coworkers demonstrated a direct relationship between the activity of aldosterone and Ang II in vascular smooth muscle cells. They found that aldosterone upregulates Ang II membrane receptors, thereby increasing the synthesis of mostol-1,4,5-triphosphate and release of intracellular Ca++. This upregulation was inhibited to a considerable extent by spironolactone, suggesting that it was primarily mediated by the mineralocorticoid receptor. These findings are consistent with a synergistic interaction between Ang II and aldosterone in the production of vascular pathology, which was first proposed by Masson and coworkers. Thus, an interaction between Ang II and aldosterone may be important in the production of end-organ damage in SHRSP.

In summary, chronic treatment with the mineralocorticoid receptor antagonist, spironolactone, markedly diminished proteinuria, renal lesions of malignant nephrosclerosis and signs of stroke in saline-drinking SHRSP. Spironolactone treatment in these animals had little or no effect on systemic arterial blood pressure or water and electrolyte excretion. These results suggest that aldosterone, or a related factor with mineralocorticoid activity, plays a major role in the development of vascular injury in saline-drinking SHRSP.

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References
12 Foster RH, MacFarlane CH, Buttamante MO. Recent progress in understanding aldosterone secretion. Gen Pharmacol 1997,28 647–651
18 Otaka J, Fujishima M, Takami K, Nakamoto Y, Ishimasa T, Omae T. Stroke-prone spontaneously hypertensive rats as an experimental model of...
Mineralocorticoids in Stroke-Prone SHR


MacLeod AB, Vasdev S, Smeda JS. The role of blood pressure and aldosterone in the production of hemorrhagic stroke in angiotensin II hypertensive rats. *Stroke* 1997, 28, 1821-1829

Tobun L, Lange J, Ulm K, Wold L, Iwa J. Potassium reduces cerebral hemorrhage and death rate in hypertensive rats even when blood pressure is not lowered. *Hypertension* 1985, 7(Suppl II), 114


Funder JW. Aldosterone, salt and cardiac fibrosis. *Clin Exp Hypertens* 1997, 19, 885-899


Gomez-Sanchez EP. Intracellular ventricular infarction of aldosterone induces hypertension in rats. *Endocrinology* 1986, 118, 819-823


Tobin L, Lange J, Ulm K, Wold L, Iwa J. Potassium reduces cerebral hemorrhage and death rate in hypertensive rats even when blood pressure is not lowered. *Hypertension* 1985, 7(Suppl II), 114


Funder JW. Aldosterone, salt and cardiac fibrosis. *Clin Exp Hypertens* 1997, 19, 885-899


Gomez-Sanchez EP. Intracellular ventricular infarction of aldosterone induces hypertension in rats. *Endocrinology* 1986, 118, 819-823


Tobin L, Lange J, Ulm K, Wold L, Iwa J. Potassium reduces cerebral hemorrhage and death rate in hypertensive rats even when blood pressure is not lowered. *Hypertension* 1985, 7(Suppl II), 114


Funder JW. Aldosterone, salt and cardiac fibrosis. *Clin Exp Hypertens* 1997, 19, 885-899
