Renal Thrombotic Microangiopathy in a Genetic Model of Hypertension in Mice

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Our goal was to develop a model of accelerated hypertension with renal microangiopathy. Transgenic mice that are hypertensive because of overexpression of human renin (R^+ mice) and human angiotensin (A^+ mice) genes were studied. To increase arterial pressure to levels comparable to those that may be seen in malignant hypertension, high salt was added to the diet and/or the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine methylester (L-NAME), was added to the drinking water. Renal lesions, decline in renal function, and proteinuria developed within 10 weeks in R^+/A^+ mice given both L-NAME and a high-salt diet, and within 24 weeks in mice given either L-NAME or a high-salt diet. Renal morphology showed features of severe thrombotic microangiopathy, with extensive vascular and glomerular lesions in all R^+/A^+ mice on high salt, L-NAME, or high salt plus L-NAME. Vascular lesions included fibrin thrombi and onion skinning of the vessel walls, whereas glomerular lesions included segmental sclerosis, mesangiolysis, fibrin thrombi within glomerular capillaries, and double-contour formation of glomerular capillary walls. Renal morphology was normal in control mice fed high salt and/or L-NAME. No R^+/A^+ mice fed a normal diet developed vascular lesions, whereas a few mice developed mild focal glomerular lesions. In summary, these studies characterize vascular and glomerular lesions in R^+/A^+ mice fed high salt, L-NAME, or both high salt and L-NAME, and provide a murine model of malignant hypertension with renal thrombotic microangiopathy. Exp Biol Med 231:196–203, 2006

Key words: hypertension; renal thrombotic microangiopathy; renin angiotensin; transgenic mice

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Hypertension is a leading risk factor for heart disease, stroke, and renal failure (1). Hypertension is the primary cause of end-stage renal disease in almost 30% of patients undergoing dialysis (2). Chronic hypertension leads to gradually progressive vascular sclerosis, interstitial fibrosis, and glomerulosclerosis. In contrast, malignant hypertension produces changes of arteries and arterioles that are more acute, with hyperplastic arteriosclerosis (onion skinning) and fibrinoid necrosis; and glomerular changes, such as mesangiolysis, fibrin in glomerular capillaries, and double-contour formation of the capillary walls (3).

The process of malignant hypertension often begins with severe renal microvascular injury, but the etiology and pathophysiology are not entirely clear. A mouse model of malignant hypertension would facilitate understanding of the causes and treatment of renal failure associated with malignant hypertension.

The renin angiotensin system modulates arterial pressure (4, 5). Transgenic mice that express human renin and angiotensin genes (R^+/A^+ mice) are chronically hypertensive, with a mean arterial pressure of approximately 150 mm Hg (6–9). Despite severe hypertension, survival of R^+/A^+ mice is normal, and only modest renal lesions are noted after many months. We developed a regimen to increase arterial pressure, with the goal of increasing blood pressure to levels that often are seen in malignant hypertension. We fed R^+/A^+ mice with a high-salt diet or added N^{ω}-nitro-L-arginine methylester (L-NAME), an inhibitor of nitric oxide synthases, to the drinking water, or administered both high salt and L-NAME. In this study, we characterized the renal lesions in the mice, and we present a model for malignant hypertension with proteinuria and severe vascular and glomerular lesions.

Materials and Methods

Animals. The experimental protocol was approved by the University of Iowa Animal Care and Use Committee. All breeding and genotyping was performed in the transgenic animal facility (directed by C.D.S.), located in a virus- and pathogen-free animal care facility.

Double transgenic mice (R^+/A^+) were generated by crossbreeding human renin (R^+) mice and human angiotensinogen (A^+) mice (6). R^+/A^+ mice develop hypertension with elevated plasma angiotensin II.

There are no differences in blood pressure between nontransgenic (R^-/A^-) and single transgenic mice (R^+/A^- or R^-/A^+), owing to the strict species specificity in the enzymatic reaction between renin and angiotensinogen. Because mouse renin does not cleave human angiotensinogen and human renin does not cleave mouse angiotensinogen, R^-/A^-, R^+/A^-, or R^-/A^+ mice were all used as controls in the present study.

Protocols. Female R^+/A^+ ($n = 31$) and control mice ($n = 28$ each) at the age of 4–5 months were divided into four groups: (i) 8% high-salt diet and normal drinking water ($n = 8$); (ii) normal diet and 100 to 120 mg L-NAME/kg/day in drinking water ($n = 8$); (iii) high-salt diet and L-NAME in drinking water ($n = 8$); and (iv) normal diet (R^+/A^+ mice or control mice, $n = 5$). Three R^+/A^+ and three control mice on normal diet (untreated) at the age of 5 months were used as baseline for renal histology. Some of the mice in this study were used in a study of spontaneous hemorrhagic stroke (10).

In R^+/A^+ mice that were fed high salt, body weight tended to greater (22 g) than R^+/A^+ mice fed either L-NAME (20 g) or a high-salt diet and L-NAME (19 g).

All eight R^+/A^+ mice fed L-NAME plus a high-salt diet died within 10 weeks. Five of 16 mice fed either L-NAME or high salt died within 24 weeks. All remaining mice received 24 weeks of treatment. After 24 weeks of each treatment, all other mice were euthanized with intraperitoneal administration of 150 mg/kg pentobarbital, and blood was collected for measurement of plasma creatinine. The kidneys were preserved in 10% formaldehyde for light microscopy, and the pole of the kidneys was preserved in 2.5% glutaraldehyde for ultrastructural analyses by electron microscopy. Formalin-fixed tissue was embedded in paraffin, and 4-μm sections were stained with hematoxylin-eosin, periodic acid Schiff (PAS), and Masson trichrome stain for histologic analysis. A minimum of 75 glomeruli and 10 vessels were examined per kidney.

The kidneys were examined blinded, without knowledge of the experimental conditions. The extent of lesions was calculated as a percentage of glomeruli and vessels that were affected by the thrombotic microangiopathic lesions. Glomeruli and arteries were considered involved when they showed one or more features of thrombotic angiopathy involving the glomeruli or arteries (see next paragraph).

Severity of the vascular lesions was graded in the involved vessels based on the extent of vascular sclerosis, onion skinning, and fibrin thrombi present within the vascular lumen. Moderate- and small-sized arteries and arterioles were examined. Vascular lesions involving less than 25% of the vessels were scored as 1, involving 25%–50% of the vessels as 2, involving 50%–75% as 3, and involving more than 75% of the vessels as 4.

Severity of glomerular lesions was graded in involved glomeruli based on the extent of segmental sclerosis, fibrin thrombi, mesangial proliferative features, and double-contour formation. Glomerular lesions involving less than 25% of the glomeruli were scored as 1, involving 25%–50% of the glomeruli as 2, involving 50%–75% as 3, and involving more than 75% of the glomeruli as 4.

Renal Function. Mice were housed in individual metabolic cages and 24-hr urine was collected. In R^+/A^+ mice fed high salt and L-NAME, urine was collected after 4 weeks of treatment. Urine in this group was collected at 4 weeks instead of 23 weeks because the mice died in the following weeks. In R^+/A^+ mice fed high salt or L-NAME treatment and in control mice fed high salt, L-NAME, or both high salt and L-NAME, urine was collected after 23 weeks of treatment. In R^+/A^+ mice and control mice fed a standard diet, urine was collected at 23 weeks of age. Renal function was assessed by measurements of plasma and urinary creatinine. Creatinine clearance was used as an index of the glomerular filtration rate and was calculated by the formula: creatinine clearance = (urinary creatinine × urine volume) / plasma creatinine. Urine protein was measured using a Bio-Rad Protein Assay Kit (Hercules, CA).

Systolic Blood Pressure. Systolic blood pressure was measured by tail cuff in conscious mice that had been trained to be accustomed to measurement of blood pressure. We measured pressure at baseline and at 8 weeks after treatment in R^+/A^+ mice on the high salt plus L-NAME diet, and at 24 weeks after treatment in the other mice.

Statistics. Results are expressed as mean ± SE. Statistical significances were analyzed with one-way ANOVA followed by Bonferroni multiple comparisons post-tests. Significance was set at $P < 0.05$.

Results

Proteinuria and Decreased Renal Function in R^+/A^+ Mice. Systolic pressure was higher in R^+/A^+ mice (117 ± 2 mm Hg) than in control mice (98 ± 2 mm Hg; $P < 0.05$). Addition of high salt to the diet increased the systolic pressure in control (108 ± 3 mm Hg) and R^+/A^+ mice (127 ± 4 mm Hg). Addition of L-NAME to the drinking water increased the systolic pressure in control (121 ± 3 mm Hg) and R^+/A^+ mice (136 ± 4 mm Hg). High salt plus L-NAME increased the systolic pressure in control (124 ± 4 mm Hg) and in R^+/A^+ mice (169 ± 6 mm Hg).

The high-salt diet produced proteinuria in control and R^+/A^+ mice (Fig. 1a). L-NAME produced minimal proteinuria. R^+/A^+ mice fed both high salt and L-NAME developed marked proteinuria (Fig. 1a). Creatinine clearance tended to decrease in R^+/A^+ mice fed either high salt or L-NAME compared with control mice (Fig. 1b). In R^+/A^+ mice fed both high salt and L-NAME, there was a significant decrease in creatinine clearance.

Vascular and Glomerular Lesions in R^+/A^+ Transgenic Mice. *Extent (Percentage) of Involvement.* No vascular lesions were observed in R^+/A^+ and

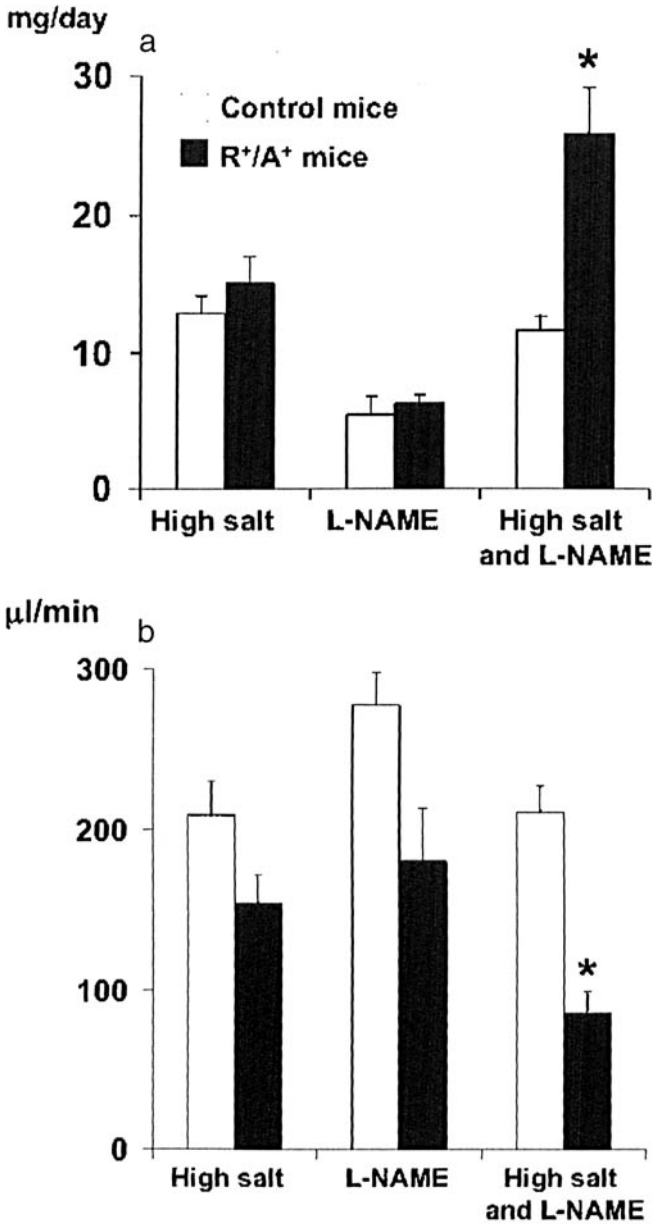

Figure 1. Renal function in control and R^+/A^+ mice. (a) Proteinuria and (b) creatinine clearance. (a) Twenty four-hour proteinuria was measured in urine of control and R^+/A^+ mice. Maximum proteinuria was noted in R^+/A^+ mice fed both high salt and L-NAME. (b) Creatinine clearance was evaluated in control and R^+/A^+ mice ($n = 28$ in R^+/A^+ mice and $n = 25$ control mice). $^*P < 0.05$, R^+/A^+ mice fed both high salt and L-NAME vs. the other groups. Data are expressed as mean ± SE.

control mice fed a normal diet. Vascular lesions were noted in all R^+/A^+ mice that were fed high salt, L-NAME, or both high salt and L-NAME (Fig. 2). The vascular lesions ranged from involvement of 31%–51% of the small- and medium-sized arteries. The extent and severity of the vascular lesions was more prominent in R^+/A^+ mice fed L-NAME or L-NAME plus high salt compared with R^+/A^+ mice fed the high-salt diet (Figs. 2 and 3a).

Glomeruli were normal in control mice. The glomerular lesions involved 31%–51% of the glomeruli in R^+/A^+ mice

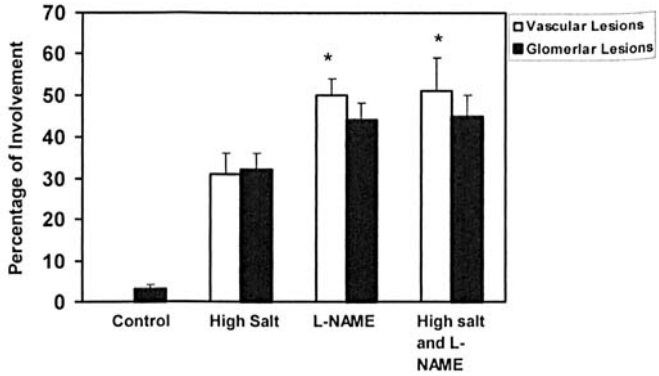

Figure 2. Extent (percentage) of glomerular and vascular involvement in R^+/A^+ mice. Seventy five glomeruli and 10 medium- and small-sized arteries were examined and the percentages of involved glomeruli and arteries were calculated (see Materials and Methods). Data are expressed as percentage ± SE. R^+/A^+ mice fed a normal diet include three mice at baseline (5 months old) and four mice after 24 weeks of a normal diet. $^*P < 0.05$, R^+/A^+ mice compared with R^+/A^+ mice fed high salt.

that were fed high salt, L-NAME, or both high salt and L-NAME (Fig. 2). Mild focal glomerular lesions were noted in R^+/A^+ mice fed a normal diet: 4 of 11 R^+/A^+ mice fed a normal diet showed mild early focal glomerular lesions (segmental glomerulosclerosis; Fig. 3b). There was no significant difference in the extent of involvement and severity of the glomerular lesions between R^+/A^+ mice fed high salt, L-NAME, or both high salt and L-NAME (Figs. 2 and 3b).

Vascular Lesions. Vascular lesions (Fig. 4) ranged from subintimal accumulation of connective tissue to onion skinning of the vascular walls to frank fibrin (thrombi) within the vascular lumen. In some vessels, the fibrin thrombi extended into the vessel wall, with rupture of the vessel wall (fibrinoid necrosis) and perivascular hemorrhage. Trichrome stains show fibrin thrombi in the walls and in the lumen of vessels. In vessels with fibrin thrombi, there was an intense mononuclear infiltrate surrounding and invading the vascular walls. Accumulation of connective tissue was also observed in the adventitia of many vessels. Vascular lesions were associated with partial to complete occlusion of the vascular lumen.

Glomerular Lesions. Glomerular lesions (Fig. 5) included segmental collapse of glomerular capillaries, with accumulation of hyaline in the subendothelial space and adhesion of the collapsed segment to Bowman's capsule (segmental sclerosis). Some glomerular capillaries contained fibrin microthrombi. Glomerular capillaries were thickened, and prominent double contours were observed along the capillary wall. The mesangium was expanded, with evidence of mesangiolysis, and there were focal proliferative features caused by an increase in mononuclear cells within the mesangium and glomerular capillaries. In a few glomeruli, there was evidence of hypoperfusion, manifested by wrinkling and thickening of the glomerular capillary walls.

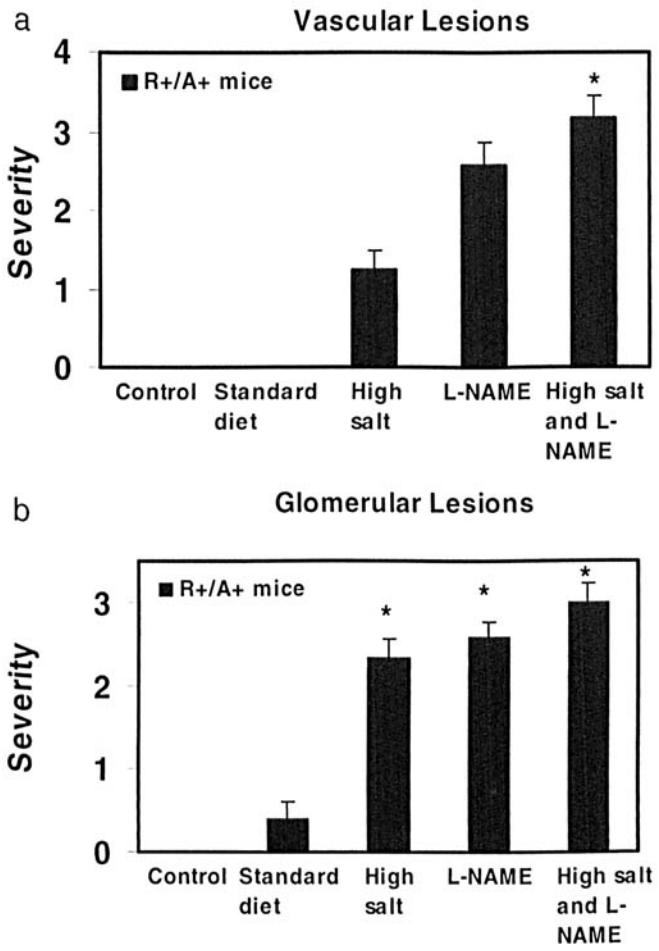

Figure 3. Comparison of treatments on severity of (a) vascular and (b) glomerular lesions in R^+/A^+ mice. The severity of the vascular and glomerular lesions was graded as described in the Materials and Methods ($n = 25$ control mice; $n = 28$ R^+/A^+ mice, with the following breakup: $n = 8$ in the high-salt group, $n = 8$ in the L-NAME group, $n = 8$ in the high salt plus L-NAME group, and $n = 5$ in the R^+/A^+ and control mice fed standard diet group). (a) $^*P < 0.001$ compared with R^+/A^+ mice fed high salt; (b) $^*P < 0.00001$ compared with R^+/A^+ mice fed standard diet.

Tubules and Interstitium. Tubules appeared normal in control mice. Many tubules were dilated and contained PAS-positive casts in R^+/A^+ mice fed high salt, L-NAME, or high salt plus L-NAME. Some tubules contained necrotic debris in the lumen (acute tubular necrosis). There was also a mild-to-moderate interstitial fibrosis present in R^+/A^+ mice fed high salt, L-NAME, or high salt plus L-NAME.

Ultrastrucure of Glomerular Capillary Walls: Remodeling with Formation of Double Contours. Examination of glomerular capillaries in R^+/A^+ mice by electron microscopy showed endothelial swelling with loss of endothelial fenestrations and expansion of the subendothelial space by cellular elements and electron lucent material. In many glomerular capillaries, there was formation of a new glomerular basement membrane beneath the cellular elements and electron-lucent material, which resulted in the formation of double contours (Fig. 6). There was focal effacement of foot processes of the visceral epithelial cells. Platelets were adherent to the endothelial cells. The mesangium was slightly expanded, with an increase in mesangial matrix and a few mononuclear cells. Fibrin thrombi were present in few glomerular capillaries. Severe glomerular capillary wall changes were noted in R^+/A^+ mice fed high salt and L-NAME, whereas lesions were less severe in R^+/A^+ mice fed either high salt or L-NAME.

Discussion

This report describes a model of severe hypertension that results in renal thrombotic microangiopathy within a few weeks of the onset of hypertension. Renal vessels and glomeruli are both involved extensively. Changes in the kidney of these mice are similar to those in patients with malignant hypertension.

Characteristics of the Transgenic Mice. In R^+/A^+ transgenic mice, expression of human renin and angiotensinogen genes is increased in many tissues, including brain, kidney, and liver (4). The mice have 4-fold higher plasma angiotensin II levels than normal mice, and are chronically hypertensive, with a mean arterial pressure of approximately 150 mm Hg (5–8). We observed that R^+/A^+ mice fed high salt and L-NAME developed augmented hypertension. Previously, we showed that, in the R^+/A^+ transgenic mice, the human renin gene is not appropriately regulated in response to angiotension II, which normally would feed back to inhibit the gene (11).

Single transgenic (R^+/A^- or R^-/A^+) mice and nontransgenic mice (R^-/A^-) are normotensive and have normal angiotensin II in plasma, because mouse renin does not cleave human angiotensinogen and human renin does not cleave mouse angiotensinogen (5). Thus, we used normotensive nontransgenic or single transgenic mice as controls.

Other Experimental Models. Tsukuba-hypertensive mice (THM) are another model of transgenic mice that overexpress human renin and human angiotensinogen, and renal lesions have been described in THM (12–15). It is of interest that when THM are fed a high-salt diet, most mice die from a rupture of an aortic aneurysm. Renal lesions in THM fed a high-salt diet are similar to those described here in R^+/A^+ mice fed a high salt (without L-NAME). In THM, the renal lesions are less severe and take almost 12–18 months to develop. Renal function was not evaluated in THM with renal lesions, and no studies have described effects of L-NAME in THM.

Bohlender *et al.* (16, 17) also studied double-transgenic rats with human angiotensinogen and renin that were hypertensive and died within 2 months. Renal vascular sclerosis was observed, but thrombi were not observed in vessels, and the glomeruli did not have lesions (16). The rats also may be a model for gestational hypertension (17).

Dahl-salt sensitive hypertensive rats and salt-fed spontaneously hypertensive rats (SHR) also develop renal disease. In those models in rats, the renal pathology is more comparable to renal changes in age-associated renal injury with uncontrolled essential hypertension, with development

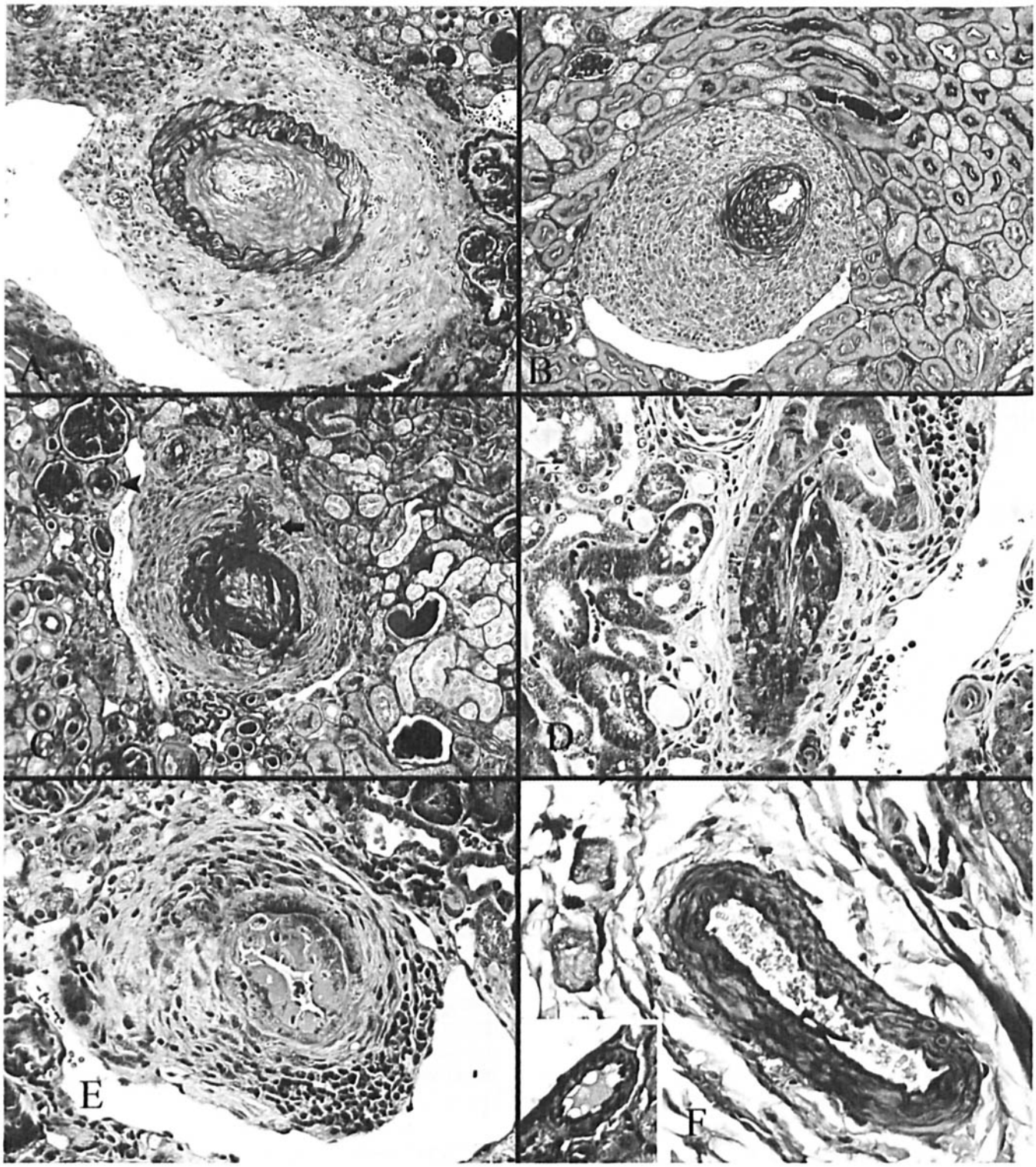

Figure 4. Sections showing typical vascular lesions in R^+/A^+ mice. The kidneys were harvested from R^+/A^+ mice after 24 weeks of treatment with high salt or L-NAME, or after 10 weeks of high salt plus L-NAME. (A) Sclerosis of the subintima and adventitia. (B) Onion skinning of the vessel wall, with inflammatory infiltrate surrounding the vessel. (C) Fibrinoid necrosis extending into the vessel wall (arrow) and fibrin thrombi in arterioles (arrowhead). (D) Fibrinoid necrosis within the wall of an artery (transmural). (E) Subendothelial expansion by fluffy granular material, narrowing of the lumen, focal fibrinoid necrosis with arterial wall, onion skinning, and mild inflammation around the vessel wall. (F) An artery from a control mouse after 24 weeks of L-NAME and high-salt treatment shows no significant abnormality (inset shows a normal arteriole). PAS stain in all figures, except D and E (trichrome stain).

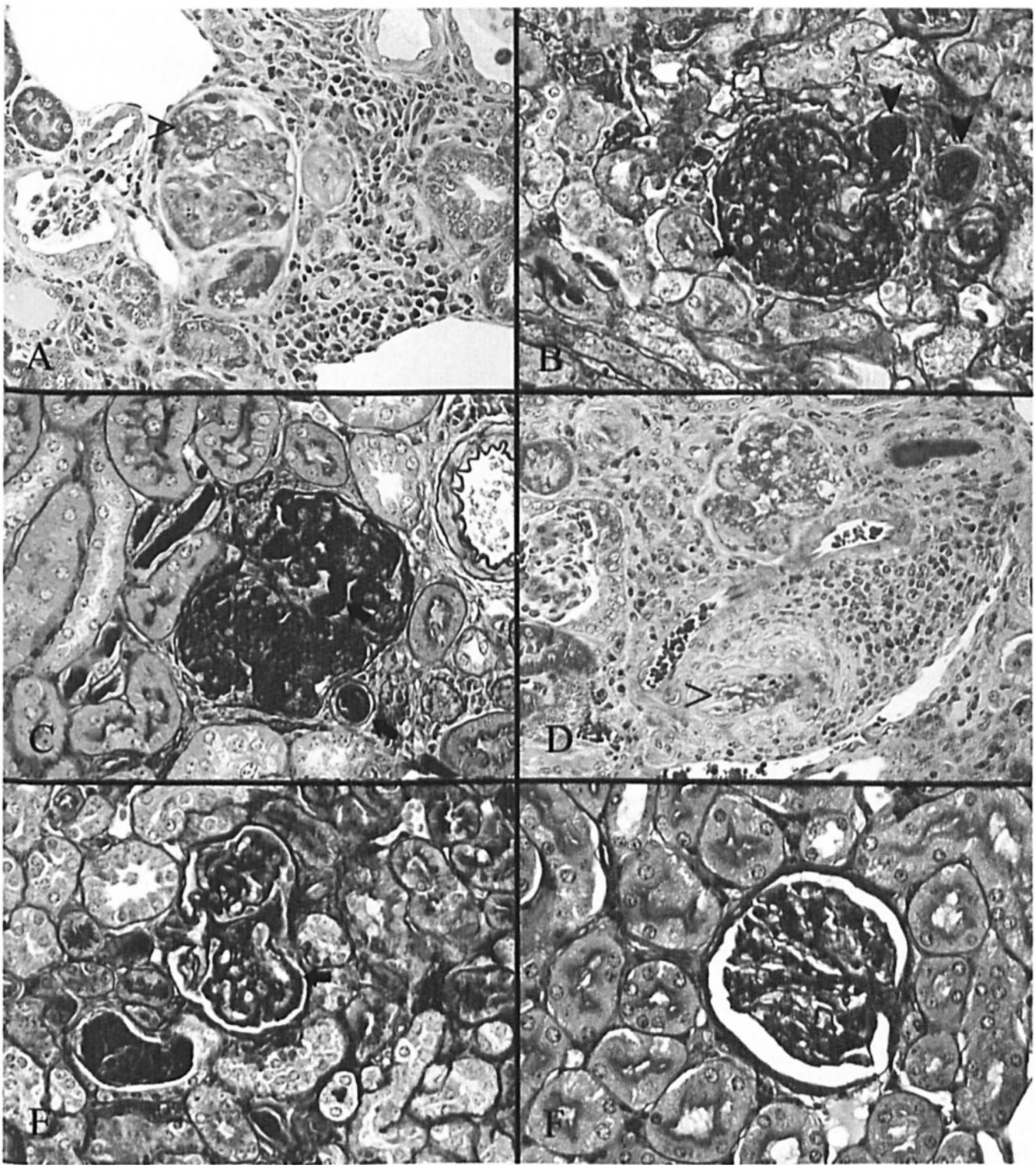

Figure 5. Sections showing typical glomerular lesions in R^+/A^+ mice. The kidneys were harvested from R^+/A^+ mice after 24 weeks of treatment with high salt or L-NAME, or after 10 weeks of high salt plus L-NAME. The glomeruli showed: (A) Fibrin microthrombi (arrowhead) in glomerular capillaries. (B) Thick capillary walls with formation of double contours (arrow) and fibrin in arterioles (arrowhead). (C) Fibrin in glomerular capillaries (arrow) with evidence of mesangiolysis. An adjacent arteriole is seen plugged with fibrin thrombi (arrow). (D) Fibrin thrombi in glomerular capillaries. Note fibrin thrombi in the vessel below (arrowhead). (E) Evidence of hypoperfusion with thickening and wrinkling of the glomerular capillary walls (arrow). (F) Glomeruli from control mice after 24 weeks of high salt plus L-NAME treatment show no significant abnormality. PAS stain in all figures, except A and D (trichrome stain).

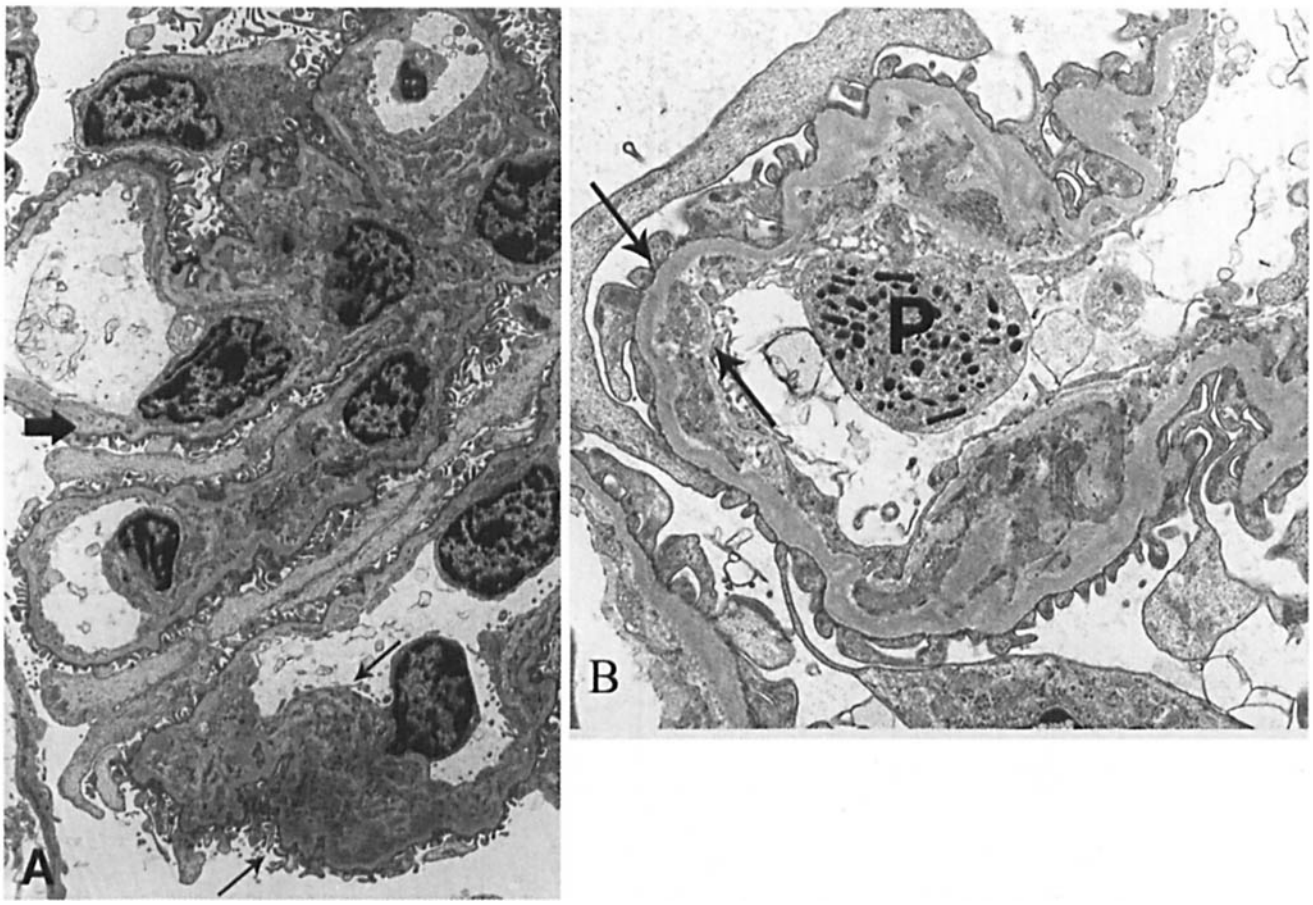

Figure 6. Electron microscopy showing glomerular capillary wall changes in R^+/A^+ mice. The glomerular capillary wall shows subendothelial expansion with fluffy granular electron-lucent material (thick arrows), and formation of double contours, with subendothelial expansion, cellular interposition, and formation of a new glomerular basement membrane (thin arrows).

of focal segmental and global glomerulosclerosis, with tubulointerstitial fibrosis, but without features of severe acute thrombotic microangiopathy (18–22). SHR fed L-NAME develop severe nephrosclerosis with features of thrombotic microangiopathy (23). SHR fed a high-salt diet also develop severe hypertension, with thrombotic microangiopathy and proteinuria, and die of strokes. In this model, aldosterone plays a pivotal role in the development of thrombotic microangiopathy, independent of the severity of the hypertension (24, 25).

Compared with these models, the lesions in our model more closely mimic the renal lesions seen in malignant hypertension for the following reasons: (i) the lesions develop over a shorter duration of time; (ii) the lesions are more extensive and involve both vessels and glomeruli; and (iii) histologically, the lesions are similar to those seen in malignant hypertension.

Histologic Changes. R^+/A^+ mice fed high salt, L-NAME, or high salt plus L-NAME develop severe changes in renal arterioles and glomeruli similar to those seen in malignant hypertension in humans. It is important to point out that severe lesions were seen in R^+/A^+ mice fed high salt plus L-NAME, and that these lesions were observed within 10 weeks after beginning treatment. Extensive lesions were also seen in R^+/A^+ mice fed either high salt or L-NAME, although these changes usually took 24 weeks to develop. Hence, the time point of urine collection was different in the three groups. In contrast, R^+/A^+ mice fed a normal diet showed no vascular lesions and only mild glomerular lesions, because 4 of 11 mice showed focal segmental sclerosing lesions that involved less than 25% of the total glomeruli. These findings are in accordance with findings in Tsukuba-hypertensive mice fed a normal diet, which develop glomerulosclerosis in approximately 10% of the glomeruli at the age of 12–18 months (12–15).

Glomerular and vascular lesions were not seen in control mice fed high salt, L-NAME, or both high salt and L-NAME. The finding is of interest because there was a modest increase in blood pressure in these mice, particularly in control mice fed high salt plus L-NAME. It is possible that these mice will gradually develop glomerular and vascular lesions if followed for a longer period. On the other hand, R^+/A^+ mice fed high salt, L-NAME, or high salt plus L-NAME rapidly develop glomerular and vascular lesions within 10–24 weeks.

We speculate that the primary mechanism by which high salt and L-NAME produce renal lesions is through augmentation of the increase in blood pressure in R^+/A^+ mice. The rapid increase in blood pressure may result in

dysfunction or injury to endothelial cells (15), which, in turn, may expose the subintimal connective tissue and activate the thrombotic cascade, which results in a prothrombotic state. We cannot exclude the possibility, however, that inhibition of nitric oxide synthases or direct effects of high salt on renal blood vessels may contribute to the development of renal lesions. On the other hand, the high-salt diet or the inhibition of nitric oxide synthases may have a synergistic effect with the renin angiotensin system in increasing the blood pressure.

Functional Change in the Kidneys. Renal function was impaired in R^+/A^+ mice fed either high salt, L-NAME, or high salt plus L-NAME, with the greatest reduction in creatinine clearance in R^+/A^+ mice fed both high salt and L-NAME. The decrease in creatinine clearance in the three treatment groups correlated with the extent of glomerular injury and vascular injury. On the other hand, there was little relationship between 24-hr proteinuria and the extent of glomerular and vascular injury. Although severe proteinuria was noted in R^+/A^+ mice with both high salt and L-NAME treatment, the extent of proteinuria was greater in R^+/A^+ mice treated with high salt than in R^+/A^+ mice treated with L-NAME.

In summary, we present a model for severe hypertension in mice with rapid development of renal morphologic features similar to those noted in malignant hypertension in humans, accompanied by proteinuria and decline in renal function. Renal morphology showed vascular lesions that were characterized by endothelial swelling, onion skinning of the intima, and media and fibrinoid necrosis within the lumen and wall of the vessels. Glomerular lesions were characterized by segmental sclerosis, mesangiolysis, and fibrin thrombi in glomerular capillaries. This model may be useful in studies of mechanisms that lead to and prevent the development of renal vascular and glomerular lesions.

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