Kidney International, Vol. 53 (1998), pp. 617–625

Angiotensinogen gene null-mutant mice lack homeostatic regulation of glomerular filtration and tubular reabsorption

Soichiro Okubo, Fumio Niimura, Taiji Matsusaka, Agnes Fogo, Brigid L.M. Hogan, and Iekuni Ichikawa

Departments of Pediatrics, Pathology, Cell Biology and Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Angiotensinogen gene null-mutant mice lack homeostatic regulation of glomerular filtration and tubular reabsorption. Chronic volume depletion by dietary salt restriction causes marked decrease in glomerular filtration rate (GFR) with little increase in urine osmolality in angiotensinogen gene null mutant ($Agt^{-/-}$) mice. Moreover, urine osmolality is insensitive to both water and vasopressin challenge. In contrast, in normal wild-type ($Agt^{+/+}$) mice, GFR remains remarkably constant and urine osmolality is adjusted promptly. Changes in volume status also cause striking divergence in renal structure between $Agt^{-/-}$ and $Agt^{+/+}$ mice. Thus, in contrast to the remarkably stable glomerular size of $Agt^{+/+}$ mice, glomeruli of $Agt^{-/-}$ mice are atrophied during a low salt and hypertrophied during a high salt diet. Moreover, the renal papilla, a structure unique to mammals and essential for urine diluting and concentrating mechanisms, is hypoplastic in $Agt^{-/-}$ mice. Thus, angiotensin is essential for the two fundamental homeostatic functions of the mammalian kidney, namely stable GFR and high urine diluting and concentrating capacity during alteration in extracellular fluid (ECF) volume. This is not only accompanied by angiotensin's tonic effects on renal vasomotor tone and tubule transporters, but also accomplished through its capacity to affect the structure of both the glomerulus and the papilla directly or indirectly.

The kidney of mammals is characterized by high regulatory capacity of tubules to reabsorb water while maintaining remarkably constant glomerular filtration rate (GFR). Teleologically, the former is believed to reflect the inconstant availability of drinking water in mammalian life. In contrast to mammals, many non-mammalian vertebrate species have highly labile GFR. In these species, GFR serves primarily as an extracellular fluid (ECF) volume regulator [1]. Thus, an acute perturbation in volume will modulate glomerular perfusion, while chronic perturbation will stimulate adjustments in glomerular size. Indeed, euryhaline fish adjust their glomerular size when they migrate between sea and fresh water [2]. Of note, non-mammalian vertebrates can afford to have highly labile GFR since toxic nitrogenous wastes are eliminated through circulatory systems independently of glomerular circulation, such as renal tubules and gills. By contrast, in mammals, nitrogenous wastes are eliminated nearly entirely through the glomerulus. Therefore, mammals, unlike other vertebrates, must maintain a constant GFR regardless of ECF volume status in order to rid the body of toxic wastes.

Angiotensin (Ang) II contributes to these features of mammalian kidneys through its constrictive action on renal arterioles [3–5] and its regulatory effect on ion transports [6–8]. With regard to the efferent arteriolar constrictive action of angiotensin II, Guyton wrote, ". . . this provides a means for conserving water and ions despite the fact that urea continues to be excreted. . . " [9]. Apart from this classic view of angiotensin action, recent studies indicate that angiotensin may promote hypertrophy and proliferation of some renal cells [10–12].

Most recent DNA recombinant studies [13–18] have demonstrated that angiotensin is involved in the ontogeny of renal papilla, a structure unique to mammals and essential for their characteristically high water reabsorptive capacity. Thus, mutant mice homozygous for the null mutation of angiotensinogen were found to have severely hypoplastic papilla. These animals are now shown by the present study to have urine dilution and concentration defects during experimental ECF volume expansion and depletion, respectively. Clearly, therefore, Ang II promotes fluid reabsorption in mammalian kidneys in part through its papilla development promoting effect.

Given the importance of Ang II in maintaining GFR in mammals [19, 20], the present study also tested the possibility that Ang II may exert another unique function in mammalian kidneys, namely, to protect glomeruli from changing size during variation in ECF volume, which occurs in non-mammalian kidneys [21]. The present study, indeed, found that Ang II maintains glomerular size in mammals by abrogating the powerful influence of extracellular fluid volume on glomerular structure. This preservation of glomerular size enables mammals to effectively remove, entirely through the glomerulus, the large amounts of nitrogenous wastes that are constantly produced independent of their hydration status.

METHODS

Mice

Wild-type mice and angiotensinogen deletion homozygous littermates were used in this study. These are the offspring of the angiotensinogen deletion mutants generated earlier by gene targeting in our laboratory [15].

The mice used in the present study were genotyped by Southern blot analysis of tail DNA. Three micrograms of tail DNA were

Key words: angiotensinogen gene, homeostasis in GFR, glomerular filtration rate, tubular reabsorption, salt restriction, diet and GFR.

Received for publication June 20, 1997
and in revised form September 8, 1997
Accepted for publication September 24, 1997

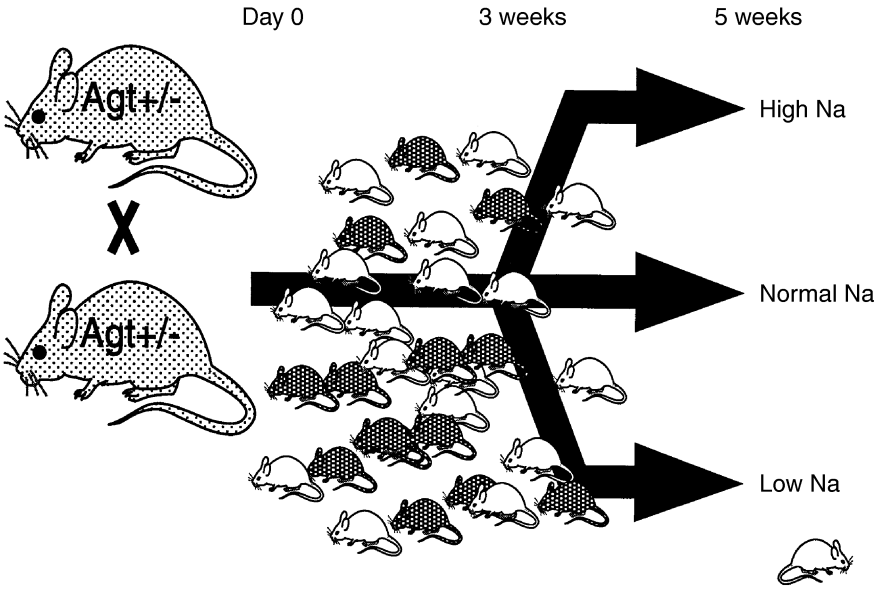

Fig. 1. The protocol for dietary regimens used in the present study. Wild-type ($Agt^{+/+}$) and homozygous angiotensinogen deletion mutant ($Agt^{-/-}$) mice were obtained by mating heterozygous ($Agt^{+/-}$) males and females. After weaning and genotying at three weeks, both $Agt^{+/+}$ and $Agt^{-/-}$ mice were fed either high, normal or low sodium-containing diets for two weeks. At the age of five weeks, they were subjected to the studies.

digested with both *Xba*I and *Xho*I, and hybridized with a ^{32}P-labeled 0.7 kb *Bgl*II-*Pst*I fragment of the angiotensinogen genomic DNA (5′ flanking probe), which was external to the 5′ end of the targeting vector. F5 mice homozygous for angiotensinogen gene null mutation ($Agt^{-/-}$) and wild-type ($Agt^{+/+}$) littermates were subjected to the analyses described below. Heterozygous littermates were not studied.

Dietary regimens

Mice were fed either a high sodium (HS) purified diet (3.15% Na; Purina Mills, Inc., St. Louis, MO, USA), low sodium (LS) purified diet (0.02% Na), or normal mouse chow [normal sodium (NS), 0.46% Na] for two weeks beginning at the time of weaning (Fig. 1). All animals had free access to food and tap water.

Blood pressure measurement

Mice (5 weeks old) were anesthetized with an intraperitoneal injection of pentobarbital (Nembutal; 50 mg/kg body wt i.p.; Abbott Laboratories., North Chicago, IL, USA) and placed on a temperature-controlled warm table. PE10 tubing, heated and tapered at one end, filled with a heparin (100 U/ml)-saline solution was inserted into the left carotid artery. The remaining portion was threaded under the skin and exited at the nape, where the end of the cannula was sealed by heating. Twenty-four hours after surgery, a piece of PE50 tubing was connected to the carotid artery cannula. The other end of the tubing was connected to a swivel to allow free mobility of the mouse. Blood pressure was measured in these conscious mice with a Cobe CDX III transducer which was connected to a Blood Pressure Analyzer (Micro-Med, Inc., Louisville, KY, USA). Blood pressure and heart rate were continuously monitored for 30 to 60 minutes until they became stable in a quiet and unrestrained environment.

Assessment of kidney function

Urine and plasma osmolality and plasma creatinine. Mice (5 weeks old) were kept in a metabolic cage (Metabolica Type MC-ST; SGI, Tokyo, Japan) for three days and urine was collected during the last 24 hours. Blood samples were then collected, and water consumption measured. Osmolality of urine and plasma was measured with an Osmette Model 5004 (Precision Systems, Inc., Sudbury, MA, USA). Plasma creatinine concentration was measured with Creatinine Analyzer 2 (Beckman Instruments, Inc., Fullerton, CA, USA).

Inulin clearance. Mice (5 weeks old) were anesthetized with an intraperitoneal injection of inactin (100 mg/kg body wt i.p.; BYK, Konstanz Germany). The PE10 tubing (Becton Dickinson and Company, Sparks, MD, USA), heated and tapered at one end, filled with heparin (100 U/ml)-saline solution, was inserted into the left carotid artery and jugular vein for blood sampling and injection of inulin. On completion of surgery, 0.1 ml of mouse plasma was infused over 10 minutes, followed by constant infusion of 4.75% inulin (USP; Iso-Tex Diagnostics., Friendswood, TX, USA) in normal saline at 0.254 ml/hr. After 60 minutes of equilibration time, three consecutive 15-minute urine collections were made. A blood sample was taken at the end of the first and third collection. Inulin concentration was measured with a standard anthrone method [22]. The renal clearance rate of inulin was calculated from a standard equation of clearance. The plasma inulin concentration of first sample, mean of first and second samples, and second sample were used for the calculation of first, second and third determination of the inulin clearance rate, respectively.

Effect of water challenge and DDAVP on urine osmolality. Protocol of this experiment was shown in Figure 2. Prior to study, the mice (5 to 6 weeks old) were maintained on normal mouse chow. For the purpose of diuresis, 5% dextrose in distilled water was given to these mice as drinking water for two days before the experiment. Mice were anesthetized with an intraperitoneal injection of inactin (100 mg/kg body wt i.p.; BYK) and placed on a temperature-controlled warm table. PE10 tubing (Becton Dickinson), heated and tapered at one end, filled with heparin (100 U/ml)-saline solution, was inserted into the left jugular vein for infusion. In order to maintain hyposthenuria during the experiment, the mice were given an infusion of hypotonic dextrose-NaCl

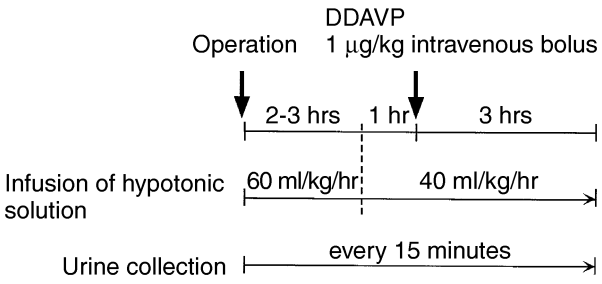

Fig. 2. Anesthetized mice, after catheterization of the left jugular vein, were given an infusion of hypotonic dextrose-NaCl solution (0.83% dextrose, 0.3% NaCl) at the rate of 60 ml/kg/hr for two to three hours. Infusion rate was then reduced to 40 ml/kg/hr for the remainder of the first study period. At the end of the first period, one hour after reduction of i.v. infusion rate, DDAVP (1 μg/kg bolus i.v., deamino-Cys[1], D-Arg[8] vasopressin) was given intravenously. Urine was collected every 15 minutes from the onset of hypotonic fluid infusion till three hours after the injection of DDAVP.

solution (0.83% dextrose, 0.3% NaCl) at the rate of 60 ml/kg/hr for two to three hours, which maintained urine osmolality at less than 172 mOsm/kg H_2O in $Agt^{+/+}$ mice. The infusion rate was then reduced to 40 ml/kg/hr for the remainder of the water diuresis period of study. One hour after the decrease of infusion speed, DDAVP (1 μg/kg bolus i.v., deamino-Cys[1], D-Arg[8] vasopressin; Sigma Chemical Co., St. Louis, MO, USA) was injected. Urine was collected every 15 minutes from the beginning of infusion until three hours after the injection of DDAVP. In $Agt^{+/+}$ mice, urine osmolality reached maximum value (more than 1000 mOsm/kg H_2O) usually one to two hours after DDAVP injection.

Light microscopic study

Kidneys were fixed in 4% buffered paraformaldehyde for 24 hours, embedded in paraffin, coronally sectioned in a thickness of 3 μm including papilla, and stained with periodic acid-Schiff. The average glomerular tuft area of all glomeruli present in the section was measured planimetrically using the Micro-Plan IITM image analysis system (Donsanto, Natick, MA, USA). All the morphological analyses were obtained by an independent pathologist without any information about the genotype of mice from which specimens were harvested.

Immunohistochemistry for α-smooth muscle actin and proliferative cell nuclear antigen

Kidneys from mice on three different diet regimens were stained for α-smooth muscle actin and proliferative cell nuclear antigen (PCNA). Horseradish peroxidase-coupled mouse anti-human α-smooth muscle actin antibody and mouse anti-human PCNA antibody (DAKO, Glostrup, Denmark) and stable DAB (a reagent with diaminobenzidine and hydrogen peroxide; Research Genetics, Inc., Huntsville, AL, USA) were used for staining.

In situ hybridization study for TGF-β1

Mouse transforming growth factor-beta1 (TGF-β1) cDNA clone (gift from Dr. H. Moses, Vanderbilt Medical School, Nashville, TN, USA) was used as a template to synthesize ^{35}S-labeled antisense and sense cRNA probe. These probes cover 1.0 kb (421-1395 bp) of mouse TGF-β1 cDNA. The protocol for *in situ* hybridization used in the current study is essentially identical to that used previously [23, 24]. Briefly, the 3-μm tissue sections were treated with 20 mg/ml proteinase K and acetic anhydride successively, dehydrated in 30 to 100% ethanol and air-dried. Sixty milliliters of hybridization mixture containing 2×10^4 cpm/ml of ^{35}S-labeled cRNA probe, 50% deionized formamide, 10% dextran sulfate, 8 mM DTT, 0.2 mg/ml tRNA and 1 × salts (300 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 0.02% polyvinylpyrolidone, 0.02% Ficoll, and 0.02% BSA) were overlaid on the specimens and incubated for 15 hours at 50°C in a humidified chamber. The specimens were washed in 50% deionized formamide, 2 × SSC, 100 mM DTT at 65°C for 20 minutes, followed by 20 mg/ml RNase at 37°C for 30 minutes. They were further washed in 2 × SSC, followed by 0.1 × SSC at 65°C, dehydrated in 30 to 100% ethanol with 0.3 M ammonium acetate, and air-dried. The slides were dipped in photographic emulsion (Ilford K-5 emulsion; Ilford Ltd., Essex, UK) and exposed at 4°C for 7 to 10 days. The sections were developed with D-19 developer (Eastman Kodak Co., Rochester, NY, USA) and counter-stained with 0.02% toluidine blue.

Plasma atrial natriuretic factor level

In order to confirm the comparably decreased state of extracellular fluid volume during low sodium diet, plasma atrial natriuretic factor (ANF) was measured in $Agt^{+/+}$ and $Agt^{-/-}$ mice on low sodium diet. Mice were decapitated and truncal blood was collected promptly into tubes kept on ice in the presence of EDTA (10 mM). The plasma was rapidly separated and kept frozen at −70°C until measurement. A commercial radioimmunoassay (RIA) (RIK9103; Peninsula Labs, Inc., Belmont, CA, USA) was used to analyze the concentration of irANF protein. Plasma irANF was measured directly on 25 μl plasma.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was assessed by analyzing variance, unpaired *t*-test and Mann-Whitney test, following ANOVA.

RESULTS

Glomerular function

As shown in Table 1, plasma creatinine concentration averaged 0.20 mg/dl ± 0.00 in $Agt^{+/+}$ mice on normal sodium diet. Value was unaffected during low sodium diet, but was slightly lower during high sodium diet. When compared to these values, those obtained in $Agt^{-/-}$ mice were some twofold higher during all three dietary regimens. Within $Agt^{-/-}$ mice, plasma creatinine level was higher during low and normal than high sodium diet, and during low than normal sodium diet.

Results of inulin clearance measurements echo these results of plasma creatinine levels. Thus, whereas values were largely unaffected by dietary sodium regimens in $Agt^{+/+}$ mice (Fig. 3, 1.17 ± 0.05 ml/min/100 g body wt, 1.04 ± 0.03, 0.97 ± 0.05 during high, normal and low sodium diets, respectively), high sodium ($P > 0.1$) increased, and low sodium ($P > 0.1$) decreased, inulin clearance in $Agt^{-/-}$ mice when compared to values during normal sodium diet (0.53 ± 0.07 ml/min/100 g body wt, 0.32 ± 0.08, 0.18 ± 0.05 during respective diets, $N = 4$ each group).

Table 1. Mean arterial pressure, water intake and whole kidney functions in $Agt^{+/+}$ and $Agt^{-/-}$ mice placed on a high, normal or low sodium-containing diet

	$Agt^{+/+}$			$Agt^{-/-}$		
	High Na	Normal Na	Low Na	High Na	Normal Na	Low Na
Urine flow rate *ml/24 hr/100 g body wt*	18.3 ± 3.2	13.1 ± 2.8	4.1 ± 1.3[a,b]	32.2 ± 4.4	28.2 ± 2.2[c]	24.3 ± 1.6[c]
Water intake *ml/24 hr/100 g body wt*	25.2 ± 5.0	15.6 ± 0.3	9.8 ± 2.6	39.0 ± 7.5	42.9 ± 2.2[c]	33.1 ± 2.7[b,c]
Plasma creatinine *mg/dl*	0.13 ± 0.03	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.30 ± 0.00[a,c]	0.43 ± 0.07[b,c]
Mean arterial blood pressure *mm Hg*	107 ± 2	105 ± 2	107 ± 1	80 ± 4[c]	70 ± 3[c]	54 ± 4[a,b,c]

The values are given as mean ± SEM ($N = 3$ mice for each parameter). Statistically significant ($P < 0.05$) compared to that of the same genotype, on high Na ([a]) or normal Na ([b]), or to that of $Agt^{+/+}$ mice on the same diet ([c]) by *t*-test (urine flow rate, water intake and mean arterial blood pressure) or by Mann-Whitney test (plasma creatinine).

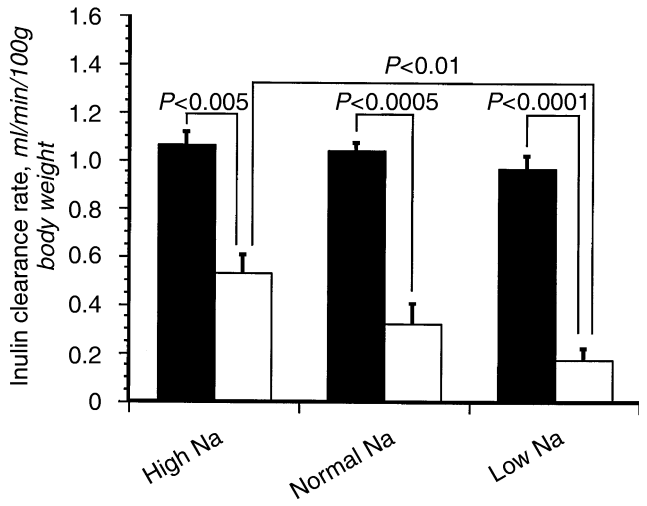

Fig. 3. Inulin clearance rate in wild-type ($Agt^{+/+}$, ■) and homozygous angiotensinogen deletion mutant ($Agt^{-/-}$, □) mice placed on three different dietary sodium regimens. Values were largely unaffected by dietary sodium in $Agt^{+/+}$ mice (1.17 ± 0.05 ml/min/100 g body wt, 1.04 ± 0.03 and 0.97 ± 0.05 during high, normal and low sodium diets, respectively). In $Agt^{-/-}$ mice, however, high sodium ($P > 0.1$) increased and low sodium ($P > 0.1$) decreased inulin clearance rate when compared to normal sodium diet (0.53 ± 0.07 ml/min/100 g body wt, 0.32 ± 0.08, and 0.18 ± 0.05 during their respective diets, $N = 4$ each group). Values are given as mean ± 1 SE.

Urine diluting and concentrating capacity

Urine flow rate was higher in $Agt^{-/-}$ than $Agt^{+/+}$ mice during all three diets (Table 1, $N = 3$ each group). Of note, in $Agt^{+/+}$ mice, the urine flow rate was significantly lower during low sodium diet when compared to that during normal ($P < 0.05$) or high sodium diet ($P < 0.05$). By contrast, no significant difference was observed among the three diets in $Agt^{-/-}$ mice. This tendency was duplicated in water intake (Table 1). Water intake was substantially higher in $Agt^{-/-}$ than $Agt^{+/+}$ mice for all three diets ($N = 3$ each group).

Urine osmolality was lower in $Agt^{-/-}$ mice when compared to that in $Agt^{+/+}$ mice (Fig. 4A), averaging 1427 ± 223 mOsm/kg H_2O, 1509 ± 158 and 3068 ± 152 during the high, normal and low sodium diets, respectively, in $Agt^{+/+}$ mice; and 934 ± 43, 644 ± 45 and 612 ± 46 on the high, normal and low sodium diets, respectively, in $Agt^{-/-}$ mice ($N = 3$ each group). The difference between $Agt^{+/+}$ and $Agt^{-/-}$ mice was statistically significant for normal and low sodium diet. Moreover, in $Agt^{+/+}$ mice, urine osmolality was higher during low sodium diet when compared to that during normal or high sodium diet. Contrastingly, in $Agt^{-/-}$ mice, urine osmolality during low sodium diet was not different from that during normal sodium diet, and, in fact, was lower than that during high sodium diet. The pattern, if any, seen in plasma osmolality was opposite to that of urine osmolality. Thus, plasma osmolality was often higher in $Agt^{-/-}$ mice than in $Agt^{+/+}$ mice (Fig. 4B; 308 ± 5 mOsm/kg H_2O, 326 ± 5 and 325 ± 1 during high, normal and low sodium diets, respectively, in $Agt^{+/+}$ mice; and 325 ± 8, 340 ± 1 and 382 ± 14 during high, normal and low sodium diets, respectively, in $Agt^{-/-}$ mice. $N = 3$ each group).

The response of urine osmolality to infusion of hypotonic solution and injection of DDAVP is shown in Figure 5. The minimum urine osmolality before the injection of DDAVP was lower in $Agt^{+/+}$ mice than $Agt^{-/-}$ mice (103 ± 17 mOsm/kg H_2O and 245 ± 47, $P < 0.05$, $N = 6$ each groups) and maximum osmolality after DDAVP injection was higher in $Agt^{+/+}$ mice than $Agt^{-/-}$ mice (1203 ± 89 and 432 ± 41, $P < 0.0001$, $N = 6$ in each group). The degree of increase in urine osmolality after DDAVP injection was higher in $Agt^{+/+}$ mice than $Agt^{-/-}$ mice (11.7 times and 1.8 times, respectively; $P < 0.001$). Continuous injection of hypotonic dextrose-NaCl solution at the rate of 60 ml/kg/hr for more than three hours caused uniform death in three $Agt^{-/-}$ mice with no more decrease in urine osmolality.

Glomerular morphology

Typical microscopic patterns seen in the superficial cortex of animals of all groups are shown in Figure 6. As shown, in $Agt^{+/+}$ mice, the size of glomeruli was not affected by dietary regimens. By contrast, in $Agt^{-/-}$ mice, the glomerular size was significantly increased during high sodium diet and decreased during low sodium diet when compared to that during normal sodium diet. As shown in Figure 7, the mean glomerular tuft area averaged 1.95 ± 0.08 mm^3 ($N = 5$), 2.01 ± 0.11 ($N = 5$) and 1.98 ± 0.09 ($N = 5$) during high, normal and low sodium diets, respectively, in $Agt^{+/+}$ mice; and 2.24 ± 0.15 ($N = 5$), 1.47 ± 0.09 ($N = 5$) and 1.06 ± 0.02 ($N = 5$) on high, normal and low Na diets, respectively, in $Agt^{-/-}$ mice. The difference between $Agt^{+/+}$ and $Agt^{-/-}$ mice was statistically significant for the normal and low sodium diets. The results of *in situ* hybridization for TGF-β1 and immunohistochemistry for α-smooth muscle actin and PCNA are shown in Figure 8. The findings of $Agt^{+/+}$ mice on high and low sodium diet were the same as normal sodium diet (not shown). The expression of mRNA of TGFβ1 was not detected in the glomeruli of both $Agt^{+/+}$ and $Agt^{-/-}$ mice on any of the three different diet regimens. The proportions of glomeruli that showed positive staining of α-smooth muscle actin in mesangial area on high,

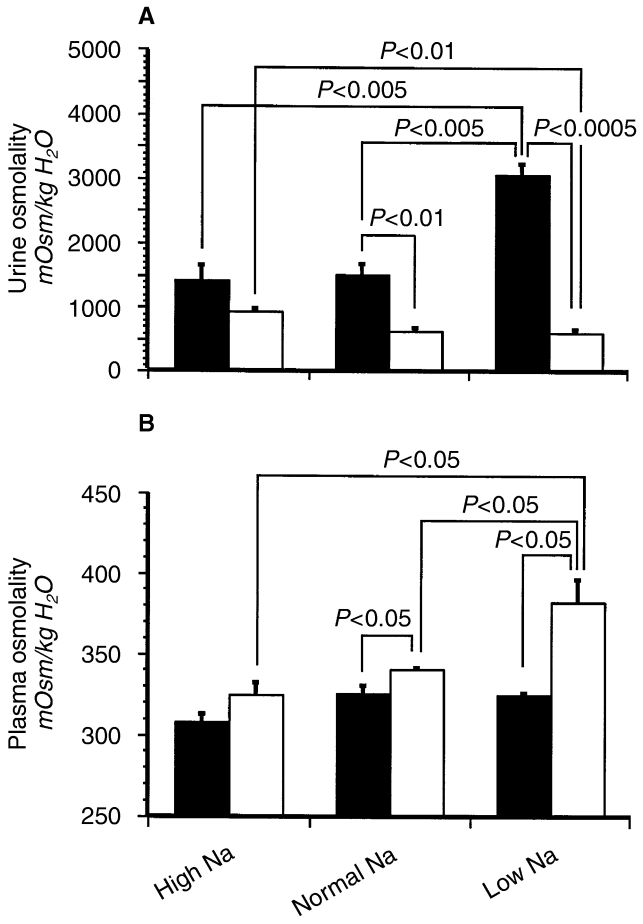

Fig. 4. Urine osmolality of wild-type ($Agt^{+/+}$, ■) and homozygous angiotensinogen deletion mutant ($Agt^{-/-}$, □) mice placed on three different dietary sodium regimens (*A*). Urine osmolality was lower in $Agt^{-/-}$ mice when compared to that in $Agt^{+/+}$ mice during high, normal and low sodium diets. **Plasma osmolality of these $Agt^{+/+}$ and $Agt^{-/-}$ mice on different sodium diets (*B*).** Plasma osmolality was often higher in $Agt^{-/-}$ mice than in $Agt^{+/+}$ mice (308 ± 5 mOsm/kg H_2O, 326 ± 5 and 325 ± 1 during the high, normal and low sodium diets, respectively, in $Agt^{+/+}$ mice; and 325 ± 8, 340 ± 1 and 382 ± 14 during the high, normal and low sodium diets, respectively, in $Agt-/-$ mice. $N = 3$ each group). Values are given as mean ± 1 SE.

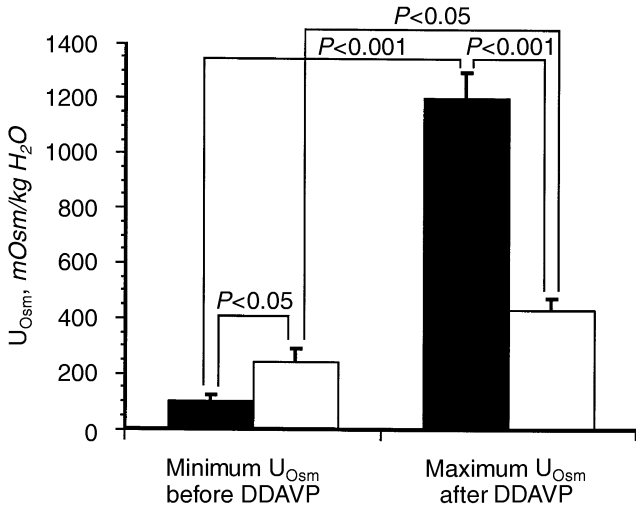

Fig. 5. Water challenge and DDAVP tests in $Agt^{+/+}$ mice (■) and $Agt^{-/-}$ mice (□). The minimum urine osmolality achieved by i.v. hypotonic fluid infusion before the injection of DDAVP was significantly lower in $Agt^{+/+}$ mice than $Agt^{-/-}$ mice (103 ± 17 mOsm/kg H_2O and 245 ± 47, $P < 0.05$, $N = 6$ each group), and the maximum osmolality after DDAVP injection was significantly higher in $Agt^{+/+}$ mice than $Agt^{-/-}$ mice (1203 ± 89 and 432 ± 41, $P < 0.0001$, $N = 6$ each group). Values are given as mean ± 1 SE.

normal and low sodium diets were 1.5 ± 1.0%, 1.8 ± 0.5 and 1.9 ± 0.5 ($N = 3$, each group), respectively in $Agt^{+/+}$ mice, and there was no statistically significant difference among the groups. In $Agt^{-/-}$ mice, these proportions on the high, normal and low sodium diets were 22.0 ± 2.3%, 14.7 ± 3.9 and 4.1 ± 1.0 ($N = 3$, each group) respectively, and significantly higher compared to those of $Agt^{+/+}$ mice on the high and normal diets ($P < 0.005$, $P < 0.05$, respectively). In $Agt^{-/-}$ mice, this proportion was significantly higher on high sodium diet compared to low sodium diet ($P < 0.001$). The proportions of glomeruli that contained PCNA positive nuclei on the high, normal and low sodium diets were 1.1 ± 0.6%, 0.7 ± 0.3 and 1.2 ± 0.6 ($N = 3$, each group) respectively in $Agt^{+/+}$ mice, and there was no statistically significant difference among the groups. In $Agt^{-/-}$ mice, these proportions on the high, normal and low sodium diets were 5.2 ± 0.8%, 6.0 ± 0.4 and 2.4 ± 0.7 ($N = 3$, each group), respectively, and significantly higher compared to those of the $Agt^{+/+}$ mice on a high sodium diet ($P < 0.05$). In $Agt^{-/-}$ mice, this proportion was significantly higher on the normal sodium diet compared to low sodium diet ($P < 0.01$).

Papillary morphology

In five-week-old $Agt^{-/-}$ mice, the pelvic space was enlarged and the papilla was uniformly hypoplastic (or atrophic) (Fig. 9). These patterns essentially duplicate those reported by us earlier from three week old $Agt^{-/-}$ mice [15].

Systemic blood pressure

Values for mean systemic arterial pressure are shown in Table 1 ($N = 3$, $N = 4$ and $N = 3$ for high, normal and low sodium diets, respectively, in $Agt^{+/+}$ mice; and $N = 3$ for all diets in $Agt^{-/-}$ mice). Values were unaffected by dietary sodium content in $Agt^{+/+}$ mice, whereas all $Agt^{-/-}$ mice were hypotensive and the level of blood pressure had a direct relationship with dietary sodium content ($P < 0.005$, $P < 0.0005$ and $P < 0.0005$ during high, normal and low sodium diets, respectively). In $Agt^{-/-}$ mice, mean blood pressure was lower during low sodium diet when compared to that during high ($P < 0.01$) or normal sodium diet ($P < 0.05$).

Plasma atrial natriuretic factor level

Plasma ANF concentration was determined after 14 days of the low sodium dietary regimen. Plasma ANF concentration was 8.2 ± 0.5 pg/100 μl ($N = 4$) in $Agt^{+/+}$ mice and 11.2 ± 1.3 ($N = 5$) in $Agt^{-/-}$ mice. There was no statistically significant difference ($P > 0.05$).

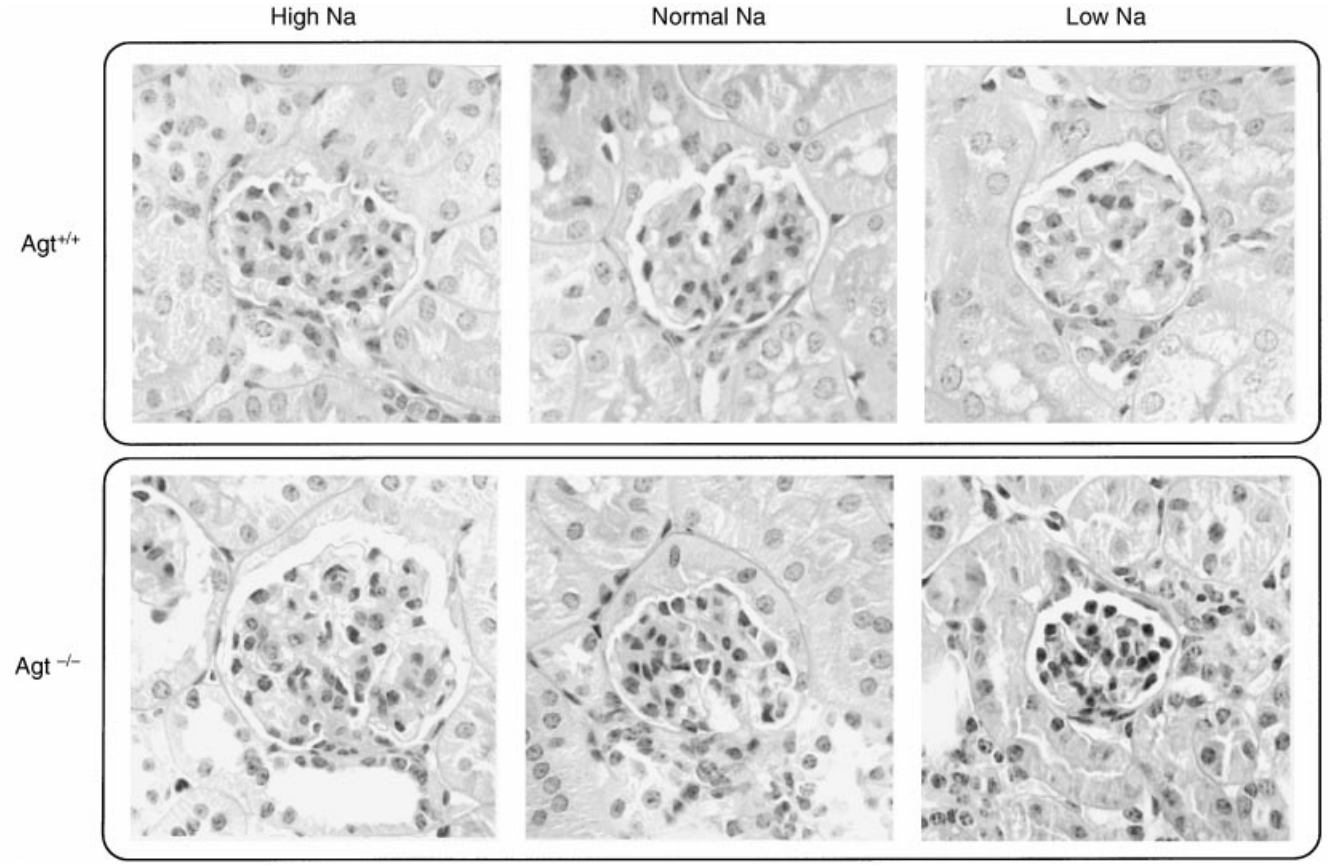

Fig. 6. Typical glomerular morphology in $Agt^{+/+}$ (top) and ($Agt^{-/-}$) mice (bottom) after two weeks of high (left), normal (center) or low (right) sodium diet regimen. The size of glomerulus was not affected by any of the diets in $Agt^{+/+}$ mice. In contrast, the glomerulus from $Agt^{-/-}$ mice placed on high sodium diet increased in size, whereas that of $Agt^{-/-}$ mice on low sodium diet decreased (Periodic acid Schiff stain, ×400).

DISCUSSION

In the present study we found that $Agt^{-/-}$ mice have severely limited capacity for both urinary dilution and concentration. Thus, during water challenge, whereas urine osmolality fell to ~100 mOsm/kg H_2O in wild-type mice, that of $Agt^{-/-}$ mice remained at ~250 mOsm/kg H_2O, or near-isotonic. In addition, a maximal dose of the vasopressin analog, DDAVP, led to an increase in urine osmolality to ~1,200 mOsm/kg H_2O in wild-type mice, whereas the urine osmolality of $Agt^{-/-}$ mice increased only slightly to ~400 mOsm/kg H_2O. Not surprisingly, therefore, during chronic ECF volume depletion with low Na diet, urine osmolality decreased (instead of increased!) to ~600 mOsm in $Agt^{-/-}$ mice, a level only ~1/5 of that achieved in $Agt^{+/+}$ mice (Fig. 4A), and ~2/3 of $Agt^{-/-}$ mice on the high Na diet. Given the notion that normal mammals undergoing severe ECF volume depletion, hypotension and 'prerenal' failure with accompanying fall in GFR concentrate their urine maximally, the observed urine concentration defect (or paradoxical fall in urine osmolality) in $Agt^{-/-}$ mice must be attributed to a mechanism that is 'intrinsically renal' in nature. These findings echo the recent observations by Esther et al [17] in mice homozygous for angiotensin I converting enzyme gene null mutation, that is, their mutants had a similar hyposthenuria. The hyposthenuria was seen in $Agt^{-/-}$ mice despite a plasma osmolality level substantially higher than that of $Agt^{+/+}$, a pattern typical for diabetes insipidus (Fig. 4B).

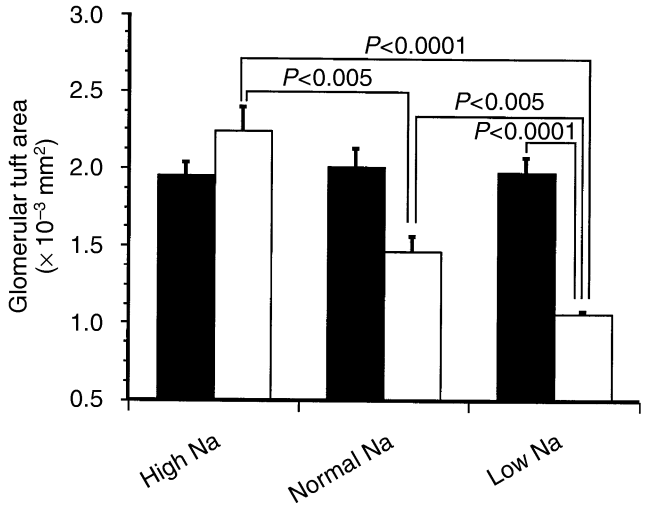

Fig. 7. Mean glomerular tuft area measured in wild-type ($Agt^{+/+}$, ■) and homozygous angiotensinogen deletion mutant ($Agt^{-/-}$, □) mice placed on three different dietary sodium regimens. The mean glomerular tuft area averaged 1.95 ± 0.08 mm^3 ($N = 5$), 2.01 ± 0.11 ($N = 5$) and 1.98 ± 0.09 ($N = 5$) during high, normal and low sodium diet, respectively, in $Agt^{+/+}$ mice; and 2.24 ± 0.15 ($N = 5$), 1.47 ± 0.09 ($N = 5$) and 1.06 ± 0.02 ($N = 5$) on high, normal and low Na diet, respectively, in $Agt^{-/-}$ mice. The difference between $Agt^{+/+}$ and $Agt^{-/-}$ mice was significant for the normal and low diets. Values are given as mean ± 1 SE.

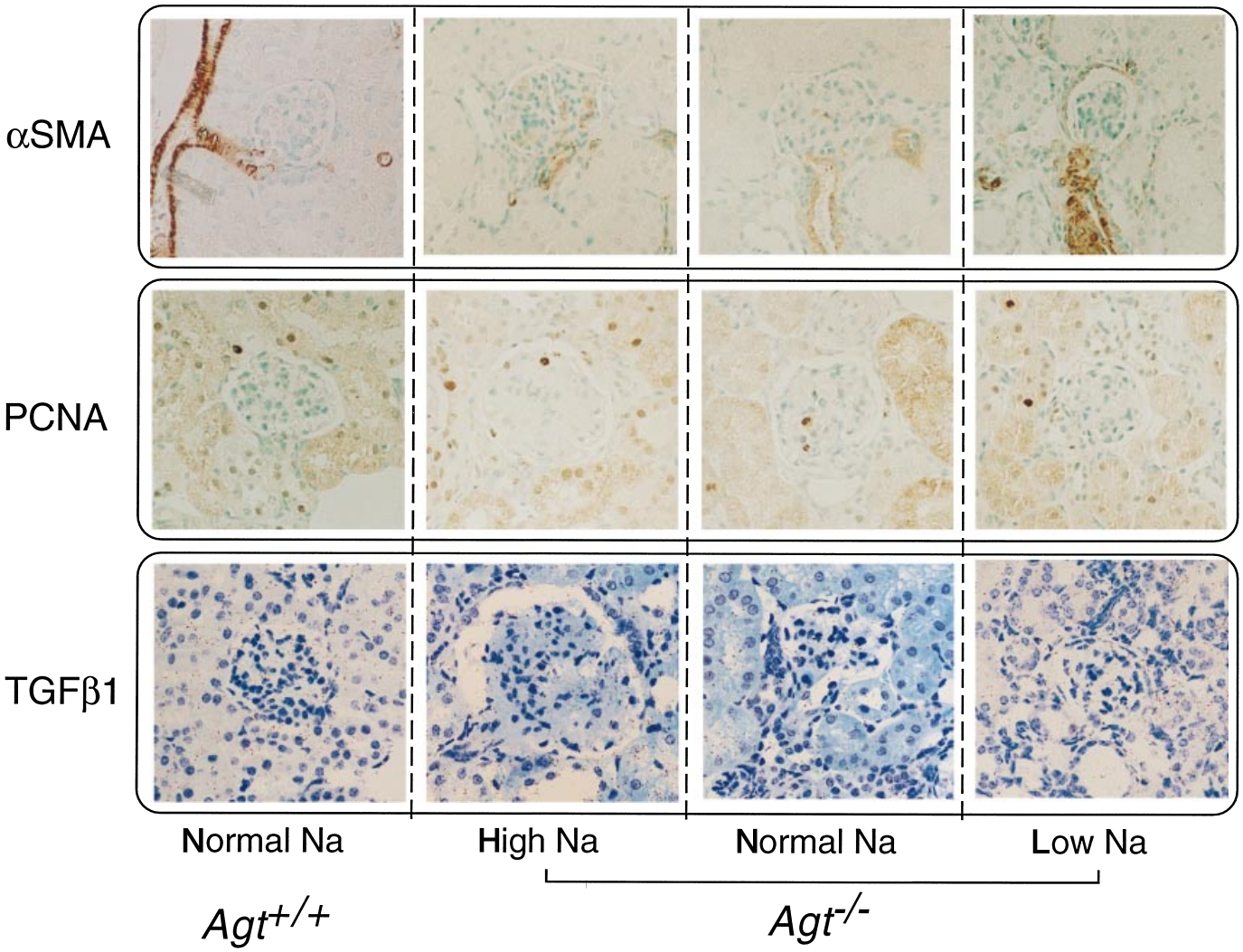

Fig. 8. Immunohistochemical studies for α-smooth muscle actin (α-SMA, brown in color) and proliferative cell nuclear antigen (PCNA, brown in color) and *in situ* hybridization studies for transforming growth factor-β1 (TGF-β1) mRNA (dark fine grains) on glomeruli from $Agt^{+/+}$ and $Agt^{-/-}$ mice fed a high, normal or low sodium-containing diet. Results from $Agt^{+/+}$ mice on high or low sodium diet are not shown as those are indistinguishable from those of $Agt^{+/+}$ mice on normal sodium diet. α-SMA positive staining within the glomerulus was more discernible in $Agt^{-/-}$ mice when they were placed on high and normal sodium diet in comparison to that on low sodium diet. PCNA positive nuclei within the glomerulus were more abundant in $Agt^{-/-}$ mice on high and normal sodium diets than on a low sodium diet. The mRNA of TGF-β1 was undetectable in the glomerulus of both $Agt^{+/+}$ and $Agt^{-/-}$ mice regardless of dietary regimen employed (×200).

The polydipsia present in $Agt^{-/-}$ mice (Table 1) is, therefore, taken to represent, at least in part, an adaptive mechanism secondary to this urine concentration defect. Since a maximal dose of AVP failed to substantially increase the urine osmolality of $Agt^{-/-}$ mice, the urine concentrating defect of these mice is attributed to an impaired responsiveness of the kidney to AVP rather than to an impaired secretory process of AVP. Of note, our present studies found that the unresponsiveness to AVP, hence impaired free water reabsorption, is accompanied by a defect in free water generation, indicating a presence of broader derangements in the counter-current system of the kidney, involving not only deep cortical but also superficial nephrons. These concurrent abnormalities at the functional level are well in concert with the abnormal morphological pattern found in $Agt^{-/-}$, specifically, hypoplastic papilla as reported earlier by us [15] and duplicated in the present study. Clearly, angiotensin is essential for the formulation of papilla, a structure unique to mammals, which enables them to regulate free water generation and reabsorption, functions essential for the volume and osmolality homeostasis of the body fluid.

The kidney of $Agt^{-/-}$ mice was found in our study to structurally and functionally resemble non-mammalian kidneys in another major way. Thus, in response to chronic manipulation of dietary salt intake, whole kidney glomerular filtration rate (GFR) estimated by plasma creatinine level during an awake condition and by inulin clearance measurement during anesthetized condition changed markedly in $Agt^{-/-}$ mice, contrasting to the GFR of $Agt^{+/+}$ mice. In the latter, GFR is, as demonstrated in the present study, characteristically insensitive to chronic manipulation of ECF volume [25, 26]. Our study further revealed that the ECF volume-sensitive GFR found in $Agt^{-/-}$ mice is accompanied by two contributory phenomena: variability in perfusion pressure and

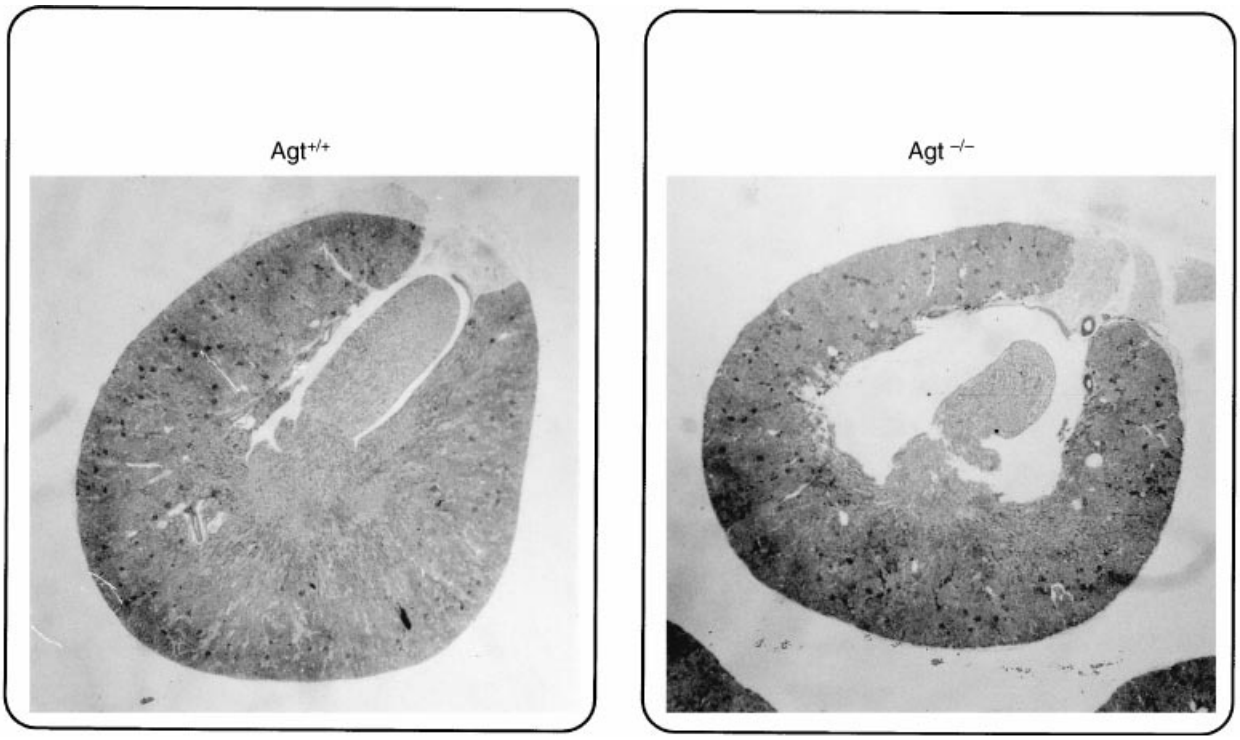

Fig. 9. Coronal sections of kidneys from five-week-old $Agt^{+/+}$ and $Agt^{-/-}$ mice. Pelvic dilation was present in $Agt^{-/-}$ mice with atrophic (or hypoplastic) papilla, contrasting to the normal appearing kidney from $Agt^{+/+}$ mice. (Periodic acid Schiff stain, ×10).

changes in glomerular size. Thus, in $Agt^{-/-}$ mice, renal perfusion pressure as reflected by systemic arterial pressure was found in our study to be highly sensitive to dietary sodium intake (Table 1). Such changes in renal perfusion pressure lead to parallel changes in GFR in the absence of adjustments of vasomotor tone within the glomerular microcirculation, which involves the constrictor action of Ang II [20]. Attenuation of the tubuloglomerular feedback may also contribute to the labile GFR, since the feedback is essential for the constancy of GFR during reduced renal perfusion pressure, and the feedback is inefficient in the absence of intact renin-angiotensin system [27]. Most remarkably, the size of glomeruli in $Agt^{-/-}$ mice was found to directly change proportionally to the amount of dietary sodium intake, although currently no technology is available to quantitate the relative importance of all these potential mechanisms for the highly ECF volume-sensitive GFR in $Agt^{-/-}$ mice. As PCNA positivity was significantly increased during high sodium diet only in $Agt^{-/-}$ mice, the increase in glomerular size in these mutants given high sodium involves cell hyperplasia. Given the expanded α-smooth muscle actin-positive area within the glomerulus of the latter, the increase in glomerular size is attributed in part, if not entirely, to mesangial cell proliferation. However, activation of TGF-β1, a well known mesangial growth factor, does not account for the proliferation, as the expression of this growth factor was not detected in any of the conditions tested. It is probable that other unmeasured growth factors, such as PDGF [28], basic FGF [29], or IGF-1 [30] are involved in the observed mesangial cell proliferation.

Overall, the functional pattern of the kidney of $Agt^{-/-}$ mice is clearly abnormal, that is, labile GFR and lack of urine concentrating capacity during changes in ECF volume. Thus, the kidney of $Agt^{-/-}$ mice has lost its fundamental homeostatic functions, that of constant elimination of nitrogeneous wastes and preservation of ECF volume. Moreover, such a loss of functional characteristics of mammalian kidneys in $Agt^{-/-}$ mice is accompanied by a loss of structural characteristics of mammalian kidneys; namely, the papilla is hypoplastic and the dimension of glomeruli now varies in accordance with the status of hydration. These observed functional and structural defects of $Agt^{-/-}$ mice are taken to reflect the renal actions of Ang II that are unique to mammals. During evolution from lower to higher vertebral species, angiotensin is believed to have progressively recruited several organs as its target tissues. These recruitments are all geared toward imparting to the animal efficient measures to meet the altered metabolic need from their new environment. It is speculated that, during evolution, angiotensin enabled the animal to progressively gain the ability to retain fluid, first through controlling glomerular perfusion and, shortly thereafter, through stimulating fluid intake via receptors in the brain. Subsequently, by stimulating tubule fluid reabsorption, either directly or through aldosterone, angiotensin further promoted fluid conservation. The results of our studies on $Agt^{-/-}$ mice indicate that, in mammals, angiotensin has recruited fourth and fifth targets, namely, the glomerulus and the papilla. These two targets are crucial for mammalian life, as they must constantly eliminate nitrogen wastes entirely through the glomerulus while preserving their hydration status, despite the highly variable availability of water in their environment.

In conclusion, while the present study provides unequivocal evidence for the dual roles of angiotensin to preserve stable GFR and to achieve a high regulatory capacity for water reabsorption [9], the study further reveals that such roles of Ang II are unique

to mammals and that both structure and function of the glomerulus and papilla are direct or indirect targets of Ang II.

ACKNOWLEDGMENTS

This work was supported by National Institute of Health grants DK-44757 and DK-37868. Dr. T. Matsusaka is the recipient of a fellowship grant from the National Kidney Foundation. Dr. Agnes Fogo is a recipient of an Established Investigator Award from the American Heart Association. We acknowledge precious advice offered by Dr. William H. Dantzler of the University of Arizona and Dr. Hiroko Nishimura of the University of Tennessee during our study.

Reprint requests to Iekuni Ichikawa, M.D., Department of Pediatrics, Vanderbilt University Medical Center, MCN C4204, Nashville, Tennessee 37232-2584, USA.
E-mail: iekuni.ichikawa@mcmail.vanderbilt.edu

REFERENCES

1. Dantzler WH: Integrative summary of renal function, in *Comparative Physiology of the Vertebrate Kidney*, edited by Dantzler WH, Berlin, 1989, Springer-Verlag, pp 157–173
2. Elger M, Hentschel H: The glomerulus of a stenohaline fresh-water teleost, Carassius auratus gibelio, adapted to saline water. *Cell Tissue Res* 220:73–85, 1981
3. Blantz RC, Konnen KS, Tucker BJ: Angiotensin II effects upon the glomerular microcirculation and ultrafiltration coefficient of the rat. *J Clin Invest* 57:419–434, 1976
4. Edwards RM: Segmental effects of norepinephrine and angiotensin II on isolated renal microvessels. *Am J Physiol* 244:F526–F534, 1983
5. Yuan BH, Robinette JB, Conger JD: Effect of angiotensin II and norepinephrine on isolated rat afferent and efferent arterioles. *Am J Physiol* 258:F741–F750, 1990
6. Harris PJ, Young JA: Dose-dependent stimulation and inhibition of proximal tubular sodium reabsorption by angiotensin II in the rat kidney. *Pflügers Arch* 367:295–297, 1977
7. Schuster VL, Kokko JP, Jacobson HR: Angiotensin II directly stimulates sodium transport in rabbit proximal convoluted tubules. *J Clin Invest* 73:507–515, 1984
8. Liu FY, Cogan MG: Angiotensin II stimulates early proximal bicarbonate absorption in the rat by decreasing cyclic adenosine monophosphate. *J Clin Invest* 84:83–91, 1989
9. Guyton AC: Renal mechanisms for concentrating and diluting the urine and for urea, sodium, potassium, and fluid volume excretion, in *Textbook of Medical Physiology* (6th ed), edited by Guyton AC, Philadelphia, WB Saunders Co., 1981, p 433
10. Johnson RJ, Alpers CE, Yoshimura A, Lombardi D, Pritzl P, Floege J, Schwartz SM: Renal injury from angiotensin II-mediated hypertension. *Hypertension* 19:464–474, 1992
11. Ray PE, Bruggeman LA, Horikoshi S, Aguilera G, Klotman PE: Angiotensin II stimulates human fetal mesangial cell proliferation and fibronectin biosynthesis by binding to AT1 receptors. *Kidney Int* 45:177–184, 1994
12. Wolf G, Neilson EG: Angiotensin II induces cellular hypertrophy in cultured murine proximal tubular cells. *Am J Physiol* 259:F768–F777, 1990
13. Tanimoto K, Sugiyama F, Goto Y, Ishida J, Yagami K, Fukamizu A, Murakami K: Angiotensinogen-deficient mice with hypotension. *J Biol Chem* 269:31334–31337, 1994
14. Kim HS, Krege JH, Kluckman KD, Hagaman JR, Hodgin JB, Best CF, Jennette JC, Coffman TM, Maeda N, Smithies O: Genetic control of blood pressure and the angiotensinogen locus. *Proc Natl Acad Sci USA* 92:2735–2739, 1995
15. Niimura F, Labosky PA, Kakuchi J, Okubo S, Yoshida H, Oikawa T, Ichiki T, Naftilan AJ, Fogo A, Inagami T, Hogan BLM, Ichikawa I: Gene targeting in mice reveals a requirement for angiotensin in the development and maintenance of kidney morphology and growth factor regulation. *J Clin Invest* 96:2947–2954, 1995
16. Nagata M, Tanimoto K, Fukamizu A, Kon Y, Sugiyama F, Yagami K, Murakami K, Watanabe T: Nephrogenesis and renovascular development in angiotensinogen-deficient mice. *Lab Invest* 75:745–753, 1996
17. Esther CR Jr, Howard TE, Marino EM, Goddard JM, Capecchi MR, Bernstein KE: Mice lacking angiotensin-converting enzyme have low blood pressure, renal pathology, and reduced male fertility. *Lab Invest* 74:953–965, 1996
18. Krege JH, John SWM, Langenbach LL, Hodgin JB, Hagaman JR, Bachman ES, Jennette JC, O'Brien DA, Smithies O: Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature* 375:146–148, 1995
19. Schnermann J, Briggs J: Role of the renin-angiotensin system in tubuloglomerular feedback. *Pediatr Proc* 45:1426–1430, 1986
20. Hall JE, Guyton AC, Jackson TE, Coleman TG, Lohmeier TE, Trippido NC: Control of glomerular filtration rate by renin-angiotensin system. *Am J Physiol* 233:F366–F372, 1977
21. Okubo S, Niimura F, Matsusaka T, Fogo A, Inagami T, Hogan BLM, Ichikawa I: Evidence from a gene targeting study that renal glomerular size is autoregulated during alteration in ECF volume in mammals due to angiotensin. (abstract) *J Am Soc Nephrol* 7:1586, 1996
22. Davidson WD, Sackner MA: Simplification of the anthrone method for the determination of inulin clearance studies. *J Lab Clin Med* 62:351–356, 1963
23. Kakuchi J, Ichiki T, Kiyama S, Hogan BLM, Fogo A, Inagami T, Ichikawa I: Developmental expression of renal angiotensin II receptor genes in the mouse. *Kidney Int* 47:140–147, 1995
24. Kiyama S, Yoshioka T, Burr IM, Kon V, Fogo A, Ichikawa I: Strategic locus for the activation of the superoxide dismutase gene in the nephron. *Kidney Int* 47:536–546, 1995
25. Steiner RW, Tucker BJ, Blantz RC: Glomerular hemodynamics in rats with chronic sodium depletion: Effect of saralasin. *J Clin Invest* 64:503–512, 1979
26. Schor N, Ichikawa I, Brenner BM: Glomerular adaptations to chronic dietary salt restriction or excess. *Am J Physiol* 238:F428–F436, 1980
27. Stowe N, Schnermann J, Hermle M: Feedback regulation of nephron filtration rate during pharmacologic interference with the renin-angiotensin and adrenergic systems in rats. *Kidney Int* 15:473–486, 1979
28. Johnson RJ, Iida H, Yoshimura A, Floege J, Bowen-Pope DF: Platelet-derived growth factor: A potentially important cytokine in glomerular desease. *Kidney Int* 41:590–594, 1992
29. Francki A, Uciechowski P, Floege J, von der Ohe J, Resch K, Radeke HH: Autocrine growth regulation of human glomerular mesangial cels is primarily mediated by basic fibroblast growth factor. *Am J Pathol* 147:1372–1382, 1995
30. Conti FG, Striker LJ, Elliot SJ, Andreani D, Striker GE: Synthesis and release of insulinlike growth factor I by mesangial cells in culture. *Am J Physiol* 255:F1214–F1219, 1988