Kidney International, Vol. 63 (2003), pp. 156–164

Potential importance of glomerular citrate synthase activity in remnant nephropathy

MICHAEL E. ULLIAN, BENJAMIN J. GANTT, AMY K. FORD, BABY G. THOLANIKUNNEL, ELEANOR K. SPICER, and WAYNE R. FITZGIBBON

The Medical University of South Carolina and The Ralph H. Johnson VA Medical Center, Charleston, South Carolina, USA

Potential importance of glomerular citrate synthase activity in remnant nephropathy.

Background. Aldosterone fosters progressive renal injury, but the mechanism is unknown. Both Wistar-Furth rats, which are resistant to aldosterone actions, and adrenalectomized Sprague-Dawley rats, which lack aldosterone, are characterized by resistance to remnant nephropathy and by reduced whole kidney citrate synthase activity. Increase in citrate synthase activity is a well-characterized, specific renal response to aldosterone. Therefore, we performed experiments to test the hypothesis that enhanced citrate synthase activity contributes to remnant nephropathy.

Methods. Rat models included Wistar (control for Wistar-Furth), Wistar-Furth (resistant to aldosterone), Sprague-Dawley (normal), adrenalectomy (lacking aldosterone), and 5/6 nephrectomy (renal injury). Glomeruli were obtained by differential sieving. Citrate synthase activity was determined spectrophotometrically. Binding characteristics of cytosolic mineralocorticoid receptors were determined by equilibrium competition binding between tritiated and unlabeled aldosterone. Gene sequencing was performed with reverse transcription-polymerase chain reaction (RT-PCR) and fluorescent dye terminators.

Results. In glomeruli isolated from adrenalectomized Wistar rats with intact renal mass, aldosterone stimulated a threefold increase in citrate synthase activity; this stimulation was not observed in glomeruli from Wistar-Furth rats. Similarly, citrate synthase activity in glomeruli isolated from adrenally intact Sprague-Dawley rats was 65% greater than that from adrenalectomized Sprague-Dawley rats. Compared to sham surgery, subtotal nephrectomy resulted in 100% greater glomerular citrate synthase activity in Sprague-Dawley rats. In Wistar-Furth rats, mineralocorticoid receptor binding was not reduced, and mutations in the mineralocorticoid receptor DNA binding segment were not found.

Conclusion. Citrate synthase activity is elevated in remnant glomeruli, and experimental models characterized by reduced glomerular citrate synthase activity (Wistar-Furth rats, adrenalectomized Sprague-Dawley rats) are protected from remnant nephropathy.

Key words: Wistar-Furth rats, mineralocorticoid receptors, aldosterone, radioligand binding, citrate synthase, remnant nephropathy, glomeruli, progressive renal injury.

Received for publication January 31, 2002
and in revised form August 2, 2002
Accepted for publication August 28, 2002

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The mineralocorticoid aldosterone may contribute to progressive renal injury. Circulating aldosterone levels are elevated in rats with experimental chronic renal failure [1, 2] and in humans with chronic renal failure [3]. Experimental nephropathy in rats can be ameliorated by adrenalectomy [4], a maneuver that eliminates circulating aldosterone, or by pharmacological blockade of aldosterone formation by treatment with an angiotensin-converting enzyme (ACE) inhibitor and an angiotensin type-1 (AT1) receptor antagonist [5, 6]. In addition, we have demonstrated that Wistar-Furth rats (WF), which are resistant to mineralocorticoid actions [1, 7–10], develop reduced renal damage after 5/6 nephrectomy [2]. Despite the increasing amount of evidence supporting a role for mineralocorticoids in progressive renal injury, the biochemical and physiological mechanisms of this mineralocorticoid action are unknown.

We hypothesize that enhanced citrate synthase (CS) activity mediates the effects of mineralocorticoids to support progressive renal injury. Activity of CS, a key Krebs cycle enzyme, is augmented specifically in the kidney by aldosterone; and, conversely, adrenalectomy, which eliminates circulating aldosterone, reduces whole kidney CS activity [11–13]. A prior study from our laboratory demonstrated reduced basal and aldosterone-stimulated CS activity in whole kidney from WF [10]. It is intriguing that WF and adrenalectomized Sprague-Dawley rats, both of which are characterized by reduced whole kidney CS activity when renal mass is intact [10–13], are resistant to renal damage after 5/6 nephrectomy [2, 4]. The present study was designed to test this hypothesis further, with special attention paid to the glomerulus, a major target of remnant injury.

METHODS

Materials

Isotopes were obtained from New England Nuclear (Boston, MA, USA). All other materials were obtained from Sigma Chemicals (St. Louis, MO, USA) except as

specified in this section. Sprague-Dawley rats, W, and WF (150 to 200 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA). RU28362 was kindly provided by Dr. D. Philibert, Roussel Uclaf (Romainville, France).

Animal surgery

The experiments described in this manuscript were conducted in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with approval of the Medical University of South Carolina Animal Care and Use Committee. Rats drank tap water ad libitum except as indicated, were fed standard rat chow (Teklad, Madison, WI, USA), and were housed in a dedicated room at a constant temperature of 25°C with a 12 hour light-dark cycle. For renal ablation, rats under pentobarbital anesthesia (50 mg/kg IP) were subjected to subtotal (5/6) renal ablation by right nephrectomy and infarction of 2/3 of the left kidney by sequential ligation of branches of the renal artery. Sham renal ablation consisted of an abdominal incision only. After adrenalectomy, accomplished via bilateral flank incisions, rats were maintained on normal chow and 1% saline to prevent extracellular fluid volume depletion.

Conscious blood pressure determinations

Conscious systolic blood pressures were measured in awake, warmed rats with a Natsume KN-2120-1 tail manometer-tachometer. Of ten blood pressure measurements obtained from each rat, the first five were discarded and the remaining data were averaged.

Cultured vascular smooth muscle cell isolation, maintenance, and characterization

Aortas from W and WF were cleaned of endothelium, fat, and adventitia for explantation. Smooth muscle strips were incubated in collagenase for two hours, cut into pieces, and allowed to adhere to a culture flask. Then, a covering layer of growth medium [10% (vol/vol) newborn calf serum, 1% (vol/vol) nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin in minimal essential medium] was added. Cells were incubated in humidified 5% CO_2/95% air atmosphere until confluent. Medium was changed every five days. Cells were passaged every seven to ten days by harvesting with trypsin-ethylenediaminetetraacetic acid (EDTA) and seeded at a ratio of 1:4. Studies were performed on cells in passages three to ten. Cells exhibited characteristic stellate morphology and stained positively for smooth muscle α-actin. Endothelial cell contamination was minimal (<3%), as assessed with antibodies to the endothelial cell marker factor VIII-related antigen.

Isolation of glomeruli

After decapsulation, renal cortices were isolated, minced in phosphate-buffered saline (PBS), and dispersed further by passage through a 90-μm sieve. The tissue was then passed through a stack of sieves (180-μm, 150-μm, 75-μm), with collection of glomeruli on the smallest. Glomeruli were washed once and then suspended in 2 mL of PBS. Purity of the glomerular preparation was confirmed by microscopy. Total number of glomeruli in the 2 mL specimen was determined by counting all glomeruli in a thoroughly suspended 10 μL sample with a hemocytometer and then multiplying by 200.

Corticosteroid radioligand binding

Cytosol as source of corticosteroid receptors was prepared from cultured vascular smooth muscle cells, kidney, or colon for ligand binding, as adapted from a previous study [14]. Cytosol sufficient for one binding study was obtained from eight serum-starved, confluent 100 mm^2 petri dishes of cultured cells or the minced cortex from one kidney or the epithelium scraped from the inside of one colon. Organs or pelleted cells were resuspended in 5 mL of lysis buffer [50 mmol/L potassium phosphate, 10 mmol/L dithiothreitol (DTT), 10 mmol/L sodium molybdate, and 1 mmol/L polymethylsulfourea, pH 7.0], homogenized with a Polytron, lysed using freeze-thaw cycles, and then further homogenized using a hand-held tissue grinder. All steps were performed strictly at 0°C. The homogenate was then centrifuged at 105,000 × *g* for 30 minutes. Supernatant was isolated and used immediately in binding studies.

Tritiated and unlabeled corticosteroids were dried under nitrogen. After 300 μL of cytosolic preparation was added, final concentration of [^{3}H]aldosterone was 50 nmol/L, and final concentration of unlabeled corticosteroid ranged in 10 increments from 0 to 100 μmol/L. Binding ensued on a rocker for one hour at 25°C. After the incubation period, bound steroid was separated from free steroid: 400 mL of a hydroxy apatite slurry (10% in lysis buffer) were added to each tube at 0°C for ten minutes, and the slurry was washed with ice-cold lysis buffer three times. The final pellet (containing bound steroid) was suspended in scintillation fluid and counted. Binding was expressed as fmol bound steroid/mg cytosolic protein. Non-specific binding was 10 to 20%.

In preliminary studies in tissues from Sprague-Dawley rats (N = 8 to 10), binding of [^{3}H]aldosterone to cytosol from homogenates of whole kidney or cultured vascular smooth muscle cells was completely inhibited by the specific glucocorticoid RU28362, suggesting that the vast majority of corticosteroid receptors in these preparations were Type II (glucocorticoid) receptors rather than Type I (mineralocorticoid) receptors. In contrast, RU28362 inhibited only a portion of binding of [^{3}H]aldosterone to cytosol from homogenates of colon, thus allowing the assessment of Type I (mineralocorticoid) receptor binding.

Renal CS activity

The formation of citrate from acetyl CoA and oxaloacetate is catalyzed by citrate synthase, with CoA-SH as a by-product. CS activity was quantitated as described previously [10] by measuring rate of generation of CoA-SH as it reacted with 5,5′-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent) to form a yellow mercaptide ion, which was quantitated spectrophotometrically. A total of 975 μL of substrate (0.25 mmol/L Ellman's reagent, 0.2 mmol/L sodium oxaloacetate, 0.1 mmol/L acetyl CoA, 100 mmol/L Tris-Cl buffer, pH 8.0) were added to 25 μL (50 μg protein) of enzyme source (described later in this section) in a 1 cm-thick cuvette at room temperature. Absorbance at 412 nm was measured every 15 seconds for five minutes. Absorbance was converted to concentration of mercaptide ion using the Lambert-Beer law (A = ecw), where A is absorbance, e is molar absorbancy index (13,600 for the mercaptide ion), c is concentration of mercaptide ion in M, and w is thickness of the reaction container in cm. The plot of product (mercaptide ion) concentration versus time was linear for the initial three to five minutes and allowed calculation of V_{max} in μmol/mg protein/min. In control studies (enzyme source with no substrate, substrate with no enzyme source), increases in absorbance were not observed.

For preparation of CS from whole kidney, one kidney was decapsulated and homogenized in buffer (0.25 mol/L sucrose, 5 mmol/L EDTA, 10 mmol/L potassium phosphate, pH 7.4). For preparation of CS from glomeruli, all glomeruli from one or two kidneys were isolated by sieving and homogenized in the same buffer. Homogenate was centrifuged at 600 rpm for 10 minutes, and the supernatant was isolated and centrifuged at 13,000 rpm for 15 minutes at 4°C. The pellet was washed once with homogenization buffer and then resuspended in 1 mL of homogenization buffer. Ten microliters of Triton X-100 were added for ten minutes at 4°C to release mitochondrial contents. Membranes were removed by centrifugation at 13,000 rpm for 15 minutes. The supernatant was isolated and the protein content adjusted to 1 mg/mL.

Mineralocorticoid receptor gene sequencing

RNA was isolated from whole kidney by the guanidinium isothiocyanate method. A 449 base-pair DNA fragment of the mineralocorticoid receptor gene, corresponding to the region coding for the DNA binding segment of the receptor (amino acids 604–659), was amplified from 1 μg of total kidney RNA by reverse transcription-polymerase chain reaction (RT-PCR). This was performed with the Access RT-PCR System (Promega, Madison, WI, USA), which uses AMV reverse transcriptase and Tf1 DNA polymerase. The following primers were used to amplify bases 1687–2136 of the coding region of the rat mineralocorticoid receptor gene [15]:

forward (24 mer)—5′-CCACACGGTGACCTGTCATCTAGG-3′
reverse (26 mer)—5′-CTTGGTCGGAGCGATGTATGTGGTCC-3′

cDNA synthesis was carried out at 48°C for 45 minutes followed by incubation at 94°C for two minutes, and then 40 cycles of three-step PCR were performed as follows: 94°C for 30 seconds, 55°C for one minute, and 68°C for two minutes. The amplified 449 base-pair fragment was visualized by electrophoresis on a 1% agarose gel and then purified on a QIAquick Spin column (Qiagen, Inc., Valencia, CA, USA). Purified polymerase chain reaction fragments were sequenced with dRhodamine fluorescent dye terminators (Perkin Elmer-ABI, Foster City, CA, USA) using an ABI 377A DNA sequencer, by the Medical University of South Carolina Biotechnology Resource Laboratory.

Protein determination

Sample protein content was determined by the Lowry method [16]. Absorbances were read at 660 nm.

Statistical considerations

Binding data were analyzed by using the Sigma Plot software program (Jandel Scientific, Corte Madera, CA, USA). Group means were compared by two-tailed, unpaired *t* test, factorial one-way analysis of variance (ANOVA), or the Wilcoxon sign rank test, where appropriate. Significant difference was assigned at the 0.05 level.

RESULTS

Effects of aldosterone on CS activity in glomeruli

Wistar-Furth and adrenalectomized Sprague-Dawley rats are protected from renal injury after subtotal renal mass reduction [2, 4]. Decreased CS activity has been observed in whole kidney preparations from WF [10] and from adrenalectomized Sprague-Dawley rats [11–13], but it is not known if the CS activity is specifically reduced in glomeruli, the main renal site in which damage occurs when renal mass is reduced. Initial studies were performed in Sprague-Dawley rats, our normal laboratory strain, to determine if increases in CS activity in response to aldosterone occur in glomeruli. Studies were performed at least seven days after bilateral adrenalectomy to eliminate the influence of endogenous mineralocorticoids. Glomeruli were isolated by differential sieving and treated ex vivo with aldosterone. Aldosterone elicited time-dependent and concentration-dependent increases in glomerular CS activity, with a maximum increase after a two-hour exposure to 1 nmol/L aldosterone (Fig. 1). Variability in basal values between animals may have obscured increases in CS activity in response to greater aldosterone concentrations.

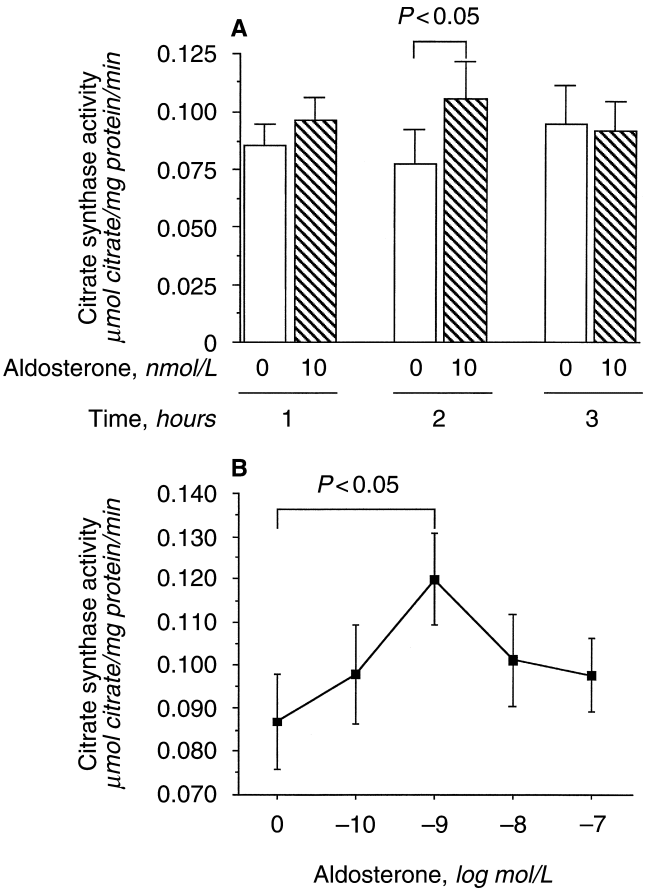

Fig. 1. Aldosterone stimulates citrate synthase (CS) activity in glomeruli ex vivo from Sprague-Dawley rats. Sprague-Dawley rats were adrenalectomized and observed for one week. Glomeruli were then isolated and pooled from both kidneys of each rat, divided into 10 aliquots of 200 μL each, and exposed to 0 or 10 nmol/L aldosterone for 1, 2, or 3 hours (*A*) or 0, 0.1, 1, 10, or 100 nmol/L aldosterone for two hours (*B*). Glomerular CS activity was measured and expressed as μmol citrate/mg protein/min. Six rats were used in Study A and 6 in Study B.

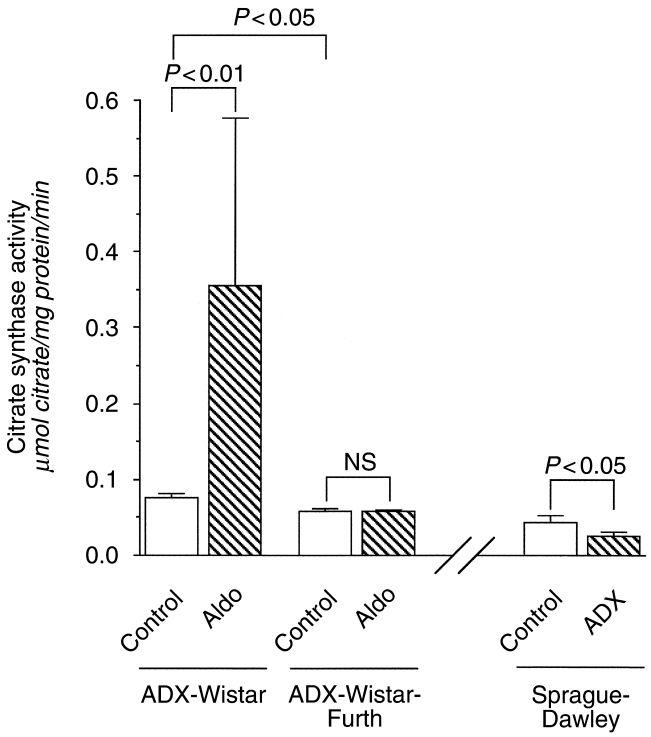

Fig. 2. Glomerular citrate synthase (CS) activity responses in adrenalectomized Wistar (W), Wistar-Furth (WF), and Sprague-Dawley rats. W and WF were adrenalectomized and observed for 1 week, and Sprague-Dawley rats were adrenalectomized or sham-operated and observed for 1 week. Glomeruli were isolated and pooled from both kidneys of each rat, divided into 8 aliquots of 200 μL each, and exposed to 0 or 1 nmol/L aldosterone for 2 hours. Glomerular CS activity was measured in duplicate aliquots for each experimental maneuver and expressed as μmol citrate/mg protein/min. Five rats from each strain were used. Since the W-WF study and the Sprague-Dawley study were not performed on the same day with the same batch of substrate, their data cannot be compared. A break in the x-axis denotes this fact. Abbreviations are: Aldo, aldosterone; ADX-adrenalectomized.

To assess the specificity of aldosterone in increasing glomerular CS activity, we exposed isolated glomeruli to angiotensin II, a potent hormone of the renin-angiotensin-aldosterone axis that is biochemical distinct from aldosterone. Glomeruli were exposed to 100 nmol/L angiotensin II for 30 minutes or two hours ex vivo, and then CS activity was measured. Angiotensin II did not increase glomerular CS activity after either of these time periods (μmol citrate/mg protein/min): 0.250 ± 0.046 (30 min vehicle) versus 0.239 ± 0.060 (30 min angiotensin II), and 0.214 ± 0.055 (2 h vehicle) versus 0.216 ± 0.070 (2 h angiotensin II; both NS, $N = 4$).

Because renal mass reduction results in fibrosis of the renal tubules and interstitium as well as glomerular sclerosis, we assessed CS activity in response to aldosterone in a tubular preparation. Tubular fragments that traversed all the sieves were exposed to 1 nmol/L aldosterone for two hours. Microscopy confirmed that this sieved fraction did not contain glomeruli. Aldosterone did not increase CS activity (μmol citrate/mg protein/min) in this tubular preparation: 0.099 ± 0.024 (control) versus 0.104 ± 0.022 (aldosterone; NS, $N = 5$). Therefore, no additional studies were performed with the renal tubular fraction. The fact that renal tubular responses of WF to aldosterone in vivo appeared to be normal [17] also directed our studies away from the tubulointerstitium and toward the glomerulus.

Once we identified the conditions under which aldosterone optimally increases CS activity in glomeruli isolated from normal (Sprague-Dawley) rats, the increases in glomerular CS activity in response to aldosterone in W were compared to those in WF. A two-hour exposure of isolated glomeruli to 1 nmol/L aldosterone elicited a threefold increase in citrate synthase activity in W but none in WF (Fig. 2). Interestingly, basal citrate synthase activity (μmol/mg glomerular protein/min) was significantly lower in glomeruli from WF (0.058 ± 0.003) com-

pared to W (0.0757 ± 0.005; $P < 0.05$). Figure 2 also demonstrates that adrenalectomy reduces glomerular CS activity in Sprague-Dawley rats by 40%. Taken together, these studies demonstrate that glomerular CS activity, like whole kidney CS activity, is significantly reduced in WF and adrenalectomized Sprague-Dawley rats.

Mechanism of reduced citrate synthase activity in WF

Abnormal renal citrate synthase activity could result from the inability of abnormal mineralocorticoid receptors to transduce the aldosterone signal or from an abnormal post-receptor signal. For our initial investigation into mineralocorticoid receptor function in WF, the ligand binding moiety of the mineralocorticoid receptor was assessed by radioligand binding studies. Cytosol was isolated from serum-deprived, cultured vascular smooth muscle cells and from kidney and colon from adrenalectomized W and WF for binding with [^{3}H]aldosterone. Serum deprivation of cultured cells and adrenalectomy in intact animals were performed to avoid prior occupancy of mineralocorticoid receptors. Our preliminary studies demonstrated that binding to mineralocorticoid receptors cannot be appreciated in vascular smooth muscle cells and whole kidney (that is, glucocorticoid receptors predominate), whereas binding to mineralocorticoid receptors can be appreciated in colonic preparations (**Methods** section). In all three tissue types, [^{3}H]aldosterone binding to cytosolic proteins was competitively inhibited by increasing concentrations of unlabeled aldosterone (Fig. 3). Qualitatively, binding was not reduced in WF compared to W in any preparation. Neither mineralocorticoid receptor density [2.3 ± 0.2 (W) vs. 4.8 ± 0.5 (WF) fmol/mg cytosolic protein, $P < 0.05$] nor binding affinity [0.25 ± 0.03 (W) vs. 0.28 ± 0.03 (WF) μmol/L] was reduced in WF compared to W. In fact, receptor density was significantly greater in WF compared to W.

To investigate a different portion of the mineralocorticoid receptor in WF, the gene sequences of the DNA binding domain of mineralocorticoid receptors from W and WF were determined. For this analysis, total RNA extracted from kidneys from W and WF was used as templates for reverse transcription and polymerase chain reaction amplification, using primers flanking the DNA binding domain of the rat mineralocorticoid receptor, that is, amino acids 604 to 659 (**Methods** section). The resulting 449 base-pair polymerase chain reaction fragments were purified (Fig. 4) and sequenced by automated fluorescent DNA sequencing techniques. The nucleotide sequences of the amplified cDNA fragments from W and WF were found to be identical to each other and to the published sequence from the normal rat [15].

Taken together, these studies demonstrate that the structure and function of the ligand-binding and DNA-binding segments of the mineralocorticoid receptor appear not to be grossly abnormal in WF. These negative studies reduced enthusiasm for further investigations of the mineralocorticoid receptor and suggest that a post-receptor aldosterone signal is abnormal.

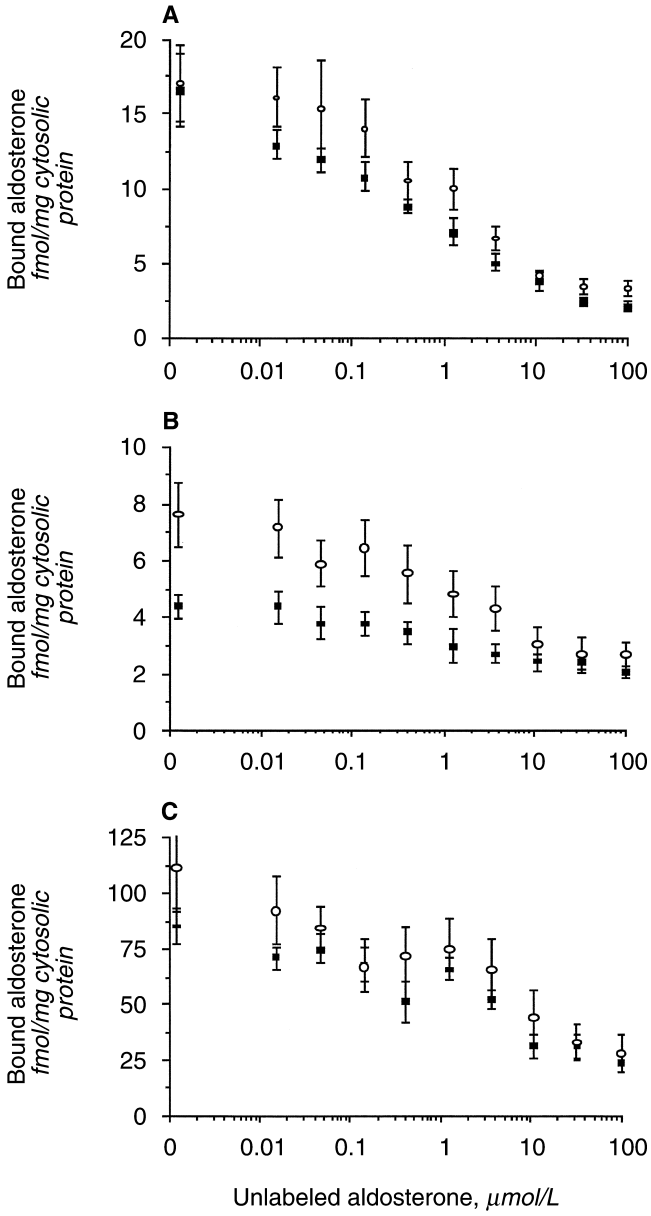

Fig. 3. Binding of [^{3}H]aldosterone to cytosol from kidney (*A*), colon (*B*), and vascular smooth muscle cells (*C*) in W (●) and WF (○). Cytosol was prepared from cultured vascular smooth muscle cells that had been serum-deprived for 24 hours and from kidney and colon from rats that had been adrenalectomized 1 week before. Competitive aldosterone binding to cytosolic constituents was performed as described in the **Methods** section. Studies in colon were performed in the presence of 1 mmol/L of the specific glucocorticoid RU28362 to allow assessment of mineralocorticoid receptors. Studies were performed 8 to 10 times for each tissue.

Glomerular CS activity in remnant kidney

In addressing the hypothesis that increased glomerular CS activity contributes to progressive renal injury, we ob-

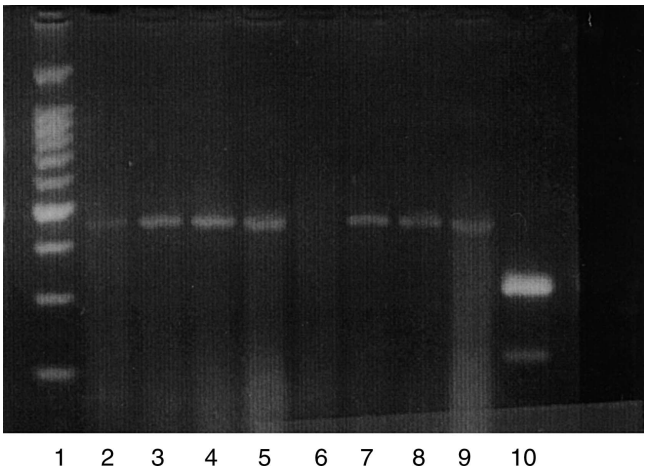

Fig. 4. Reverse transcription-polymerase chain reaction (RT-PCR) product of cDNA from the DNA-binding domain of the mineralocorticoid receptor in W and WF. RNA was isolated from whole kidney of W and WF. A 449-base-pair region spanning the DNA-binding domain of the mineralocorticoid receptor was amplified with RT-PCR, purified, and sequenced. Lane 1, standards starting at 200 base-pairs and increasing by 100; lanes 2–5, W; lanes 6–9, WF; lane 10, RNA control and primers. Differences in densities within each strain resulted from differing $MgSO_4$ concentrations.

Table 1. Somatic and renal parameters in sham-operated and 5/6-nephrectomized Sprague-Dawley rats

	Body weight	Kidney weight	Glomeruli	Albuminuria
	g		*per kidney*	*mg/day*
Sham	235 ± 4	1.057 ± 0.027	18,640 ± 900	14 ± 3
Remnant	164 ± 10[a]	1.014 ± 0.089	4,020 ± 538[a]	261 ± 37[a]

Abbreviations are: sham, sham-operated; remnant, 5/6-nephrectomized.
[a] $P < 0.01$ compared to sham

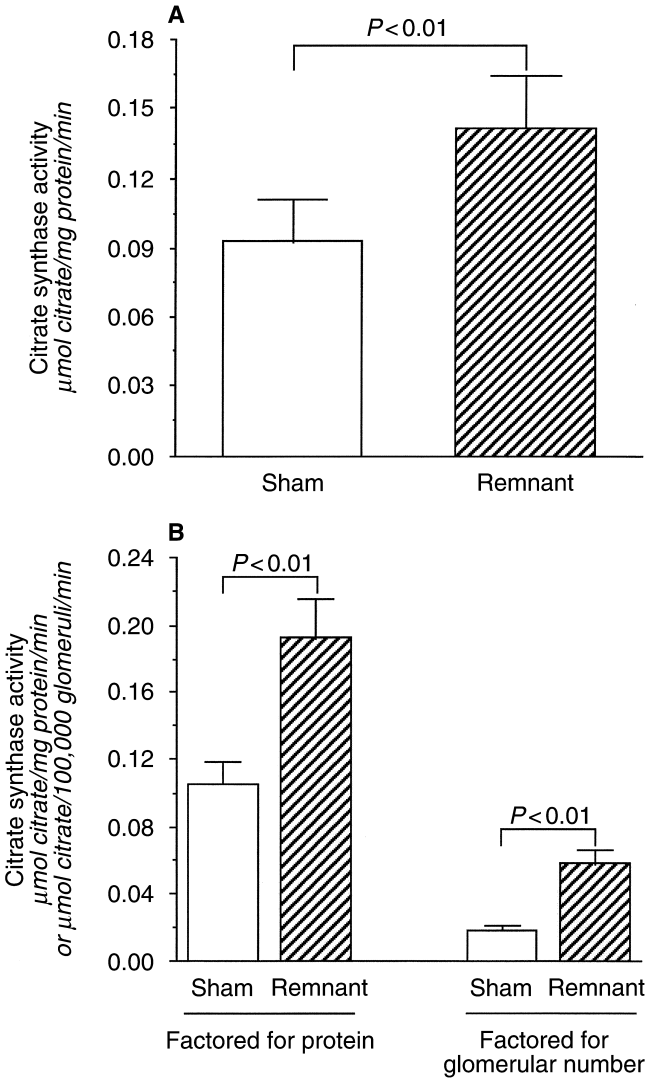

Fig. 5. CS activity in sham and remnant kidney. Sprague-Dawley rats were subjected to sham surgery or 5/6 nephrectomy and observed for 4 weeks. CS source was prepared from the left kidney from each of 9 sham-operated animals and the remnant kidney from each of 9 5/6-nephrectomized animals (*A*); and from glomeruli isolated from the left kidney from each of 10 sham-operated animals and from glomeruli isolated from the remnant kidney from each of 10 5/6-nephrectomized animals (*B*). CS activity was measured and expressed as µmol citrate/mg protein/min and µmol citrate/10^5 glomeruli/min.

served reduced whole kidney and glomerular CS activity in two experimental models (WF and adrenalectomized Sprague-Dawley) characterized by protection against renal damage when renal mass is reduced. Our next studies were designed to investigate the corollary situation, that is, that a reduction of renal mass would result in increased citrate synthase activity in the remaining kidney and glomeruli. These studies were performed in our normal laboratory rat strain, Sprague-Dawley. At four weeks after sham surgery or 5/6 nephrectomy, urine was collected in metabolic cages for 24 hours, body weights were measured, conscious blood pressure was determined, and animals were sacrificed. Table 1 demonstrates that renal mass reduction was accomplished: 5/6 nephrectomy reduced number of glomeruli per kidney by 75%, renal hypertrophy had occurred (remnant kidneys had grown to equal the weights of normal kidneys), and renal mass reduction effected renal injury (marked increase in albuminuria). Systolic blood pressure was 35 to 40 mm Hg greater in remnant animals than normal animals. Figure 5A demonstrates that CS activity was more than 50% greater in whole remnant kidney compared to whole normal kidney. The study was then repeated for measurement of glomerular CS activity. We found that CS activity was almost 100% greater in glomeruli from 5/6-nephrectomized animals compared to CS activity in glomeruli from sham-operated animals (Fig. 5B).

DISCUSSION

Prior studies have demonstrated that adrenalectomy in standard laboratory rat strains, such as Sprague-Dawley, resulted in reduced levels of circulating aldosterone,

reduced whole kidney CS activity [11–13], and reduced renal damage after renal mass reduction [4]. Similarly, WF, which are not deficient in circulating aldosterone but appear to be resistant to the actions of aldosterone at the tissue level [1, 10], are also characterized by reduced whole kidney CS activity [10] and reduced renal damage after renal mass reduction [2]. These data are consistent with the possibility that decreased renal CS activity protects against progressive renal injury. The corollary possibility is that increased CS activity contributes to progressive renal injury. Our present study was designed to gather further preliminary data in support of this hypothesis.

In whole kidney of normal rats, aldosterone activates CS, and removal of aldosterone, that is, adrenalectomy, reduces CS activity. However, we wondered if aldosterone activates CS in glomeruli as in whole kidney preparations. The answer to this question becomes relevant to studies on the role of glomerular CS activity in remnant nephropathy, because the glomerulus is a major site of injury after 5/6 nephrectomy. By studying isolated glomeruli, we eliminated the influence of circulating and hemodynamic factors. In preliminary studies in Sprague-Dawley rats, we found that aldosterone can activate glomerular CS ex vivo in a time- and concentration-dependent fashion (Fig. 1). Aldosterone also activates CS in glomeruli isolated from W (Fig. 2), just as we had found in other normal (Sprague-Dawley) rats (Fig. 1). In contrast, aldosterone was unable to elicit an ex vivo increase in CS activity in glomeruli isolated from WF (Fig. 2). In addition, basal glomerular CS activity was lower in WF compared to W and in adrenalectomized Sprague-Dawley rats compared in adrenally-intact Sprague-Dawley rats (Fig. 2). These results and results from our prior study demonstrate that WF and adrenalectomized Sprague-Dawley rats, both of which are resistant to renal damage when renal mass is subtotally ablated [2, 4], are characterized by reduced CS activity not only in the intact kidney but specifically in glomeruli from intact kidney, a major target site of renal damage when renal mass is subtotally ablated. These results are essential for support of the hypothesis that activation of CS contributes to glomerular damage after 5/6 nephrectomy. This is the first report of increases in CS activity upon exposure to mineralocorticoids in glomeruli. Prior studies have documented renal cortical collecting duct, whole kidney, and myocardium as tissues that respond to aldosterone with increases in CS activity [13]. The existence of glomerular mineralocorticoid receptors has been documented [18].

Having determined that basal and aldosterone-stimulated glomerular CS activities are reduced in WF, a strain of rat that develops a significantly lesser amount of renal damage after renal mass reduction [2], we examined the corollary hypothesis: renal mass reduction in normal rats results in increased CS activity in the remaining glomeruli. Indeed, we found this to be true (Fig. 5), regardless of how the data were expressed (μmol citrate per mg glomerular protein per minute or μmol citrate per glomerular number per minute). Taken together, the correlation of reduced CS activity in glomeruli from WF prior to renal mass reduction with reduced renal damage after renal mass reduction in WF and the increase in CS in remnant glomeruli in Sprague-Dawley rats support our hypothesis. It is possible that the increase in glomerular CS activity in remnant glomeruli is a result of the glomerular damage rather than a contributor to the glomerular damage. However, the opposite cannot be so, that is, that reduced CS activity is a result of the reduced renal damage in WF or adrenalectomized Sprague-Dawley animals, since subnormal renal CS activity exists prior to renal mass reduction in these two rat models (Fig. 2).

Although correlation of glomerular CS activity with glomerular damage over a wide range of glomerular citrate synthase activities is consistent with our hypothesis, more studies are needed to prove the hypothesis. For example, we will repeat the experiment in Figure 5, performed here with Sprague-Dawley rats, in W and WF. We predict that CS activity in glomeruli isolated from remnant kidney will be greater than CS activity in glomeruli isolated from sham-operated kidney in W, as in Sprague-Dawley rats. In WF, however, we predict that glomerular CS activity will not increase with renal mass reduction, because WF are characterized by subnormal expression of CS. The development of remnant nephropathy may depend upon the ability of CS activity to increase in the remnant glomeruli after renal mass reduction.

Involvement of increased Kreb cycle enzyme activity in the pathogenesis of remnant nephropathy has not been previously proposed. The classical theory of aldosterone's mineralocorticoid actions in the distal renal tubule consists of effects on luminal permeability (increased number or activity of epithelial sodium channels), sodium pumps (increased number or activity of Na,K-ATPase, and metabolism (activated mitochondrial enzyme activity to generate ATP) [11]. It has been suggested that heightened activity of the Kreb cycle may provide fuel for the sodium pumps to accomplish antinatriuresis. In remnant glomeruli, increases in CS activity and ATP may be necessary to fulfill the energy demands for aldosterone-induced glomerular alterations that lead to hypertrophy, hyperfiltration, and glomerulosclerosis.

Although not designed for this purpose, the present studies furnish information on mechanisms of resistance to the actions of mineralocorticoids in WF. Increased circulating aldosterone levels [1, 2] and failure to develop hypertension when mineralocorticoid and salt are administered [1, 7–10] are consistent with resistance to aldosterone action at the end organ. The inability of aldosterone to effect increases in glomerular CS activity in WF (Fig. 2) stimulated us to investigate mineralocorticoid receptor function. The mineralocorticoid receptor, the initial aspect

of the aldosterone signal transduction pathway, has been shown to be abnormal in humans with aldosterone resistance, that is, pseudohypoaldosteronism [19, 20]. We examined mineralocorticoid receptor function by molecular and biochemical techniques. Our findings—that ligand binding of aldosterone to mineralocorticoid receptors is not reduced in WF (Fig. 3) and that the gene sequence of the DNA binding domain of the mineralocorticoid receptor is not abnormal in WF—demonstrate normal structure and function of two major moieties of the mineralocorticoid receptor. Our ligand binding results are in accord with those of another group of investigators who reported that renal receptor density and binding affinity for mineralocorticoid and glucocorticoid receptors were not reduced in WF compared to W [21]. Despite our present findings of normal aldosterone ligand binding and normal gene sequence of the DNA-binding domain of the WF mineralocorticoid receptor, it is still possible that the mineralocorticoid receptor of WF is abnormal and mediates end organ resistance to mineralocorticoids. The mineralocorticoid receptor consists of specific domains necessary for ligand binding, receptor dimerization, DNA binding, nuclear translocation, and transactivation (recruiting accessory proteins so that transcription will initiate), and we must consider the possibility that an abnormality exists in a region of the WF mineralocorticoid receptor other than those investigated in the present study. Increased mineralocorticoid receptor density in WF is consistent with the interrupted negative feedback mechanisms seen in tissue resistance states.

Another consideration for the site of resistance to mineralocorticoids in WF is the corticosteroid response elements in the regulatory regions of mineralocorticoid target genes. However, this possibility is unlikely. Since enhancement of action of multiple vasoconstrictors (that is, norepinephrine, angiotensin II, serotonin), a well-known vascular action of mineralocorticoids, is attenuated in WF [1, 10], abnormalities in the regulatory regions of genes for the receptors for all those hormones would have to be postulated. The existence of such a large number of mutations is possible but unlikely.

Abnormalities in gene products induced by mineralocorticoids, so-called aldosterone-induced proteins, must be considered in WF. Early products include the more recently described small g-protein K-Ras2 and sgk (serum- and glucocorticoid-induced kinase), and later products include epithelial sodium channels, Na,K-ATPase, CS, and vasoconstrictor (angiotensin II, AT_1, α-adrenergic) receptors [22]. It is unlikely that vasoconstrictor receptors are abnormal in structure or expression, since contractile responses to vasoconstrictors in the absence of mineralocorticoids are not decreased in WF [1, 10]. Differences in epithelial sodium channel and/or Na,K-ATPase between W and WF are unlikely, because aldosterone stimulates anti-natriuresis equally well in W and WF [17] and because aldosterone stimulates hypokalemia equally well in W and WF [1, 10]. Nothing is known about the early response genes (K-Ras2 and sgk) in WF. We have demonstrated that basal CS activity and responses of CS activity to mineralocorticoids are distinctly abnormal in kidney from WF. Abnormal expression of CS may be the abnormality in the mineralocorticoid signal transduction pathway that mediates resistance to mineralocorticoids in WF.

ACKNOWLEDGMENTS

This work was supported by funds from the American Heart Association South Carolina Affiliate and Dialysis Clinic Incorporated. A portion of this work was presented in abstract form at the annual meeting of the Council for High Blood Pressure Research of the American Heart Association in Philadelphia, PA, September 1998. The authors thank Ms. Jana J. Fine and Mr. George O. Washington for technical assistance, the Medical University of South Carolina Biotechnology Resource Laboratory for DNA sequence analysis, and the Medical University of South Carolina Oligosynthesis Facility for the preparation of oligonucleotide primers.

Reprint requests to Michael E. Ullian, M.D., Division of Nephrology, Clinical Sciences Building 829, Medical University of South Carolina, 171 Ashley Avenue, Charleston, South Carolina 29425, USA.
E-mail: ullianme@musc.edu

REFERENCES

1. Bruner CA: Vascular responsiveness in rats resistant to aldosterone-salt hypertension. *Hypertension* 20:59–66, 1992
2. Fitzgibbon WR, Greene EL, Grewal JS, *et al*: Resistance to remnant nephropathy in the Wistar-Furth rat. *J Am Soc Nephrol* 10: 814–821, 1999
3. Hene RJ, Boer P, Koomans HA, Dorhout Mees EJ: Plasma aldosterone concentrations in chronic renal failure. *Kidney Int* 21: 98–101, 1982
4. Quan ZY, Walser M, Hill GS: Adrenalectomy ameliorates ablative nephropathy in the rat independently of corticosterone maintenance level. *Kidney Int* 41:326–333, 1992
5. Greene EL, Kren S, Hostetter TH: Role of aldosterone in the remnant kidney model in the rat. *J Clin Invest* 98:1063–1068, 1996
6. Rocha R, Chander PN, Zuckerman A, Stier C: Role of aldosterone in renal vascular injury in stroke-prone hypertensive rats. *Hypertension* 33:232–237, 1999
7. Jeffries WB, McArdle S, Bockman C, *et al*: Vasopressin response in collecting ducts of rats resistant to mineralocorticoid hypertension. *Hypertension* 17:63–71, 1991
8. Molteni A, Nickerson PA, Gallant S, Brownie AC. Resistance of W/Fu rats to adrenal regeneration hypertension. *Proc Soc Exp Biol Med* 150:80–84, 1975
9. Sciotti V, Gallant S: Resistance to mineralocorticoid-induced hypertensive vascular disease. *Hypertension* 10:176–180, 1987
10. Ullian ME, Islam MM, Robinson CJ, *et al*: Resistance to mineralocorticoids in the Wistar-Furth rat. *Am J Physiol* 272:H1454–H1461, 1997
11. Kirsten R, Kirsten E: Redox state of pyridine nucleotides in renal response to aldosterone. *Am J Physiol* 223:229–235, 1972
12. Law PY, Edelman IS: Induction of citrate synthase by aldosterone in the rat kidney. *J Membr Biol* 41:41–64, 1978
13. Marver D, Schwartz MJ: Identification of mineralocorticoid target sites in the isolated rabbit cortical nephron. *Proc Natl Acad Sci USA* 77:3672–3676, 1980
14. Schulman G, Miller-Diener A, Litwack G, Bastl CP: Characterization of the rat colonic aldosterone receptor and its activation process. *J Biol Chem* 261:12102–12108, 1986
15. Patel PD, Sherman TG, Goldman DJ, Watson SJ: Molecular

cloning of a mineralocorticoid (Type I) receptor complementary DNA from rat hippocampus. *Mol Endocrinol* 3:1877–1885, 1989
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275, 1951
17. Ullian ME, Robinson CJ, Evans CTB, *et al*: Role of citrate synthase in aldosterone-mediated sodium reabsorption. *Hypertension* 35:875–879, 2000
18. Todd-Turla KM, Schnermann J, Fejes-Toth G, *et al*: Distribution of MR and GR mRNA along the nephron. *Am J Physiol* 264:F781–F791, 1993
19. Armanini D, Karbowiak I, Zennaro CM, *et al*: Pseudohypoaldosteronism: Evaluation of type I receptors by radioreceptor assay and by antireceptor antibodies. *Steroids* 60:161–163, 1995
20. Zennaro MC, Borenstein P, Jeunemaitre X, *et al*: Molecular characterization of the mineralocorticoid receptor in pseudohypoaldosteronism. *Steroids* 60:164–167, 1995
21. Kayes K, Ziegler L, Yu CP, *et al*: The resistance of the Wistar/Furth rat strain to steroid hypertension. *Endocr Res* 22:681–689, 1996
22. Verrey F: Early aldosterone action: toward filling the gap between transcription and transport. *Am J Physiol* 277:F319–F327, 1999