It has been suggested that the impaired natriuretic response of the clipped kidney in two-kidney, one clip hypertensive rats is related to downregulation of renal atrial natriuretic peptide receptors. To test this hypothesis, blood volume expansion and atrial peptide binding studies were performed in this model. Infusion of 1% and then 1.5% body weight donor blood (n=6) caused a progressive increase in plasma immunoreactive atrial natriuretic peptide (107±26 to 168±31 to 427±154 pg/ml, p<0.001); the sodium excretion of the nonclipped kidney rose from 230 to 2,200 to 4,000 neq/min (p<0.01) but that of the clipped kidney did not rise significantly. There was a highly significant correlation between log cyclic guanosine monophosphate and log sodium excretion by the nonclipped (r²=0.749) but not the clipped (r²=0.046) kidney. Between clipped and nonclipped kidneys, the association constant (5.26±0.89 versus 5.17±0.64·10⁻⁷mol) and apparent binding site density (575±92 versus 500±74 fmol/mg protein) for atrial peptide binding in isolated glomeruli did not differ. Assay of atrial peptide–induced cyclic guanosine monophosphate release by isolated glomeruli showed that clipped and nonclipped kidneys were equally responsive. Binding affinity and receptor density did not differ in homogenates prepared from inner medullas of clipped and nonclipped kidneys. These results show that the blunted natriuretic response in clipped kidneys was not associated with any relative decrease in number or function of glomerular or papillary atrial natriuretic peptide receptors. (Hypertension 1991;18:535–542)

In 1950, White observed that during unilateral renal artery constriction in dogs, the kidney behind the stenosis excreted less salt and water than the contralateral kidney, even at a time when glomerular filtration rate and renal plasma flow were not affected. Based on this work, Howard et al first proposed a diagnostic test for surgically correctable renovascular hypertension based on urine sodium and volume measurements obtained by individually cannulating the ureters. The use of fractional excretion of sodium in "split renal function studies" for diagnostic purposes was later refined by Rapoport and by Birchall et al. These tests were said to be positive in the large majority of cases of unilateral main renal artery disease, although their use for screening in clinical practice was eventually abandoned because bilateral or segmental renal arterial disease was often missed, and cystoscopy was required to obtain the samples.

The two-kidney, one clip (2K1C) Goldblatt hypertensive rat, like the patient with unilateral renal artery stenosis, chronically excretes much less sodium from the stenotic than the contralateral kidney. Huang et al have demonstrated in this model that the natriuretic and diuretic response to infusion of atrial natriuretic peptide (ANP) is essentially abolished in the clipped kidney in the presence of a brisk response on the other side.

We had previously found that volume expansion of normal rats with donor blood produced a marked rise in circulating immunoreactive ANP, which correlated well with the subsequent natriuretic and diuretic responses. ANP appears to play a significant role in producing the natriuretic response to blood volume expansion, since the studies of Stasch et al and Wilkins et al have shown that administration of an anti-ANP monoclonal antibody markedly inhibits the renal response to saline loading in normal rats.

In the present study, it was observed that the response of the clipped kidney of 2K1C rats to blood volume expansion was significantly blunted compared to the nonclipped kidney. These findings suggest that the blunted natriuretic response in the clipped kidney may be due to a decrease in the number or function of atrial natriuretic peptide receptors.
volume expansion was markedly blunted compared with that of the contralateral kidney. Gauquelin and coworkers\textsuperscript{10,11} had previously examined glomerular membranes 3 weeks after clipping in 2K1C rats and found that ANP receptors in pooled membranes from clipped kidney glomeruli were decreased in number relative to nonclipped or sham-operated kidneys. Since ANP appears to be a major determinant of the renal response to blood volume expansion, the remainder of the present study was designed to investigate the hypothesis that blunted natriuresis by the clipped kidney at baseline and after blood volume expansion could be attributed in part to decreased renal ANP receptor number or function in that kidney.

Methods

All experiments were performed in 2K1C Sprague-Dawley rats. Rats weighing 100–125 g underwent sterile placement of a silver clip of 0.2 mm internal aperture on the right renal artery under pentobarbital anesthesia. All studies were performed between 3 and 4 weeks after clipping, when the rats had achieved body weights of 250–300 g.

**Acute Blood Volume Expansion Studies**

The animals were anesthetized with thiobutabarbital (100 mg/kg i.p., Inactin, By Gulden, Hanover, FRG). A tracheostomy was performed, and the internal jugular vein was cannulated for infusion of saline (0.6 ml/hr) and 10% inulin in saline (0.6 ml/hr). A femoral arterial cannula was introduced and connected to a pressure transducer (Carolina Medical Instruments, Quincy, Mass.) for continuous arterial pressure monitoring. The contralateral femoral vein was connected to a pump-mounted syringe reservoir containing approximately 12 ml donor rat blood anticoagulated with heparin. Blood volume removed for measurement of inulin, sodium, and ANP was immediately replaced from the reservoir. The ureters of the clipped and nonclipped kidneys were cannulated through a transabdominal midline incision, which was then loosely sutured shut to minimize evaporative fluid loss. At least 30 minutes were allowed after the completion of surgery for stabilization before the start of clearance measurements. Animals with a mean arterial pressure of less than 150 mm Hg at this point were discarded.

Two 20-minute baseline clearance periods were obtained. The animals then received an infusion of 1% of their body weight of donor blood over 15 minutes. After three more 20-minute clearance periods, another blood infusion of 1.5% body weight was administered over 15 minutes, and three more 20-minute clearance periods were obtained. Blood samples of 0.5 ml were drawn at the midpoint of each clearance period for inulin and sodium determinations, and samples for measurement of plasma immunoreactive ANP were drawn at the end of the control clearances, immediately after each blood volume expansion period, and at the end of the experiment. Urinary sodium and inulin concentrations were measured during each clearance period; urinary cyclic guanosine monophosphate (cGMP) excretion was measured during the clearance periods immediately before and after each blood volume expansion step.

**Glomerular and Papillar Atrial Natriuretic Peptide Binding Studies**

A separate group of 2K1C rats were killed; the kidneys were removed within 60 seconds and were placed into iced Hanks' balanced salt solution (HBSS). Glomeruli were isolated by a modification of the method of Ballermann et al.\textsuperscript{12} The entire procedure was performed on ice. The cortices of the clipped and nonclipped kidneys were removed into separate cold Petri dishes and minced in HBSS with a razor. The minced renal cortex was forced through a stainless steel sieve with a mesh aperture size of 90 μm. The tissue was rinsed from the underside of the sieve with cold HBSS, and washed through stacked 180-μm and 150-μm sieves. The filtrate was collected and passed through a 75-μm sieve.

The tissue remaining on top of the 75-μm sieve was rinsed with cold HBSS into a conical bottom tube and centrifuged for 3 minutes at 150g. The supernatant was poured off and the glomeruli were resuspended in cold HBSS. Centrifugation and resuspension were repeated twice.

After the final centrifugation the glomeruli from each kidney were resuspended in 1.2 ml cold HBSS. Two hundred microliters were removed for determination of protein concentration, and the remainder brought to 2.5 ml with HBSS containing bovine serum albumin (BSA), bacitracin, 4-[(2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES), and phenylmethyl sulfonyl fluoride (PMSF), adjusted to pH 7.4. The final composition of the buffer was: BSA 0.2%, HEPES 10 mM, PMSF 90 mg/l, and bacitracin 100 mg/l in HBSS.

The glomerular suspension obtained by this method was routinely monitored for purity by light microscopy and consisted of approximately 90% intact, decapsulated glomeruli. Occasional glomeruli retained capsules or short pieces of afferent or efferent arterioles, and occasional fragments of coiled proximal tubule were seen. Repeated counts of 10-μl aliquots of several preparations showed that they contained 2,000–3,000 glomeruli/ml.

Competitive binding studies were performed in a shaking water bath at 4°C. Each tube contained 100 μl glomerular suspension, 50 μl [125I]-ANP (about 25,000 cpm; specific activity approximately 2,000 Ci/mmol, Amersham, Arlington Heights, Ill.) and 50 μl of various concentrations of unlabeled rat ANP 1–28 (Peninsula Labs, Belmont, Calif.), and was incubated for 2 hours. At the end of incubation, glomeruli were separated from buffer by suction filtration on 25-mm glass fiber filters (Whatman GF/C, Maidstone, England) that had been pretreated by soaking in 1% polyethyleneimine for 2 hours or more. The glomeruli were washed three times within 30 seconds with 3 ml phosphate-buffered saline (PBS) containing 0.2%...
BSA, and the filters were counted in a gamma counter with 76% efficiency (Beckman Instruments, Irvine, Calif.).

A third group of 2K1C animals was used to obtain papillary tissue for radioligand binding studies. Despite multiple modifications of technique, it was never possible to obtain adequate material for generation of a complete binding curve from a single rat papilla. Consequently, the inner medullas from three to five clipped and nonclipped kidneys from the same animals were homogenized and pooled for each of four experiments, according to a modification of the method of Martin et al.13

Rats were rapidly decapitated, and the kidneys were removed into ice-cold PBS, pH 7.4. The kidneys were bisected, and inner medullas were removed with scissors. The tissue was finely minced with a razor and was subjected to hypotonic shock for 5 minutes in 5 mM ethylene diamine tetraacetic acid (EDTA) to lyse cells other than inner medullary collecting tubule cells. After centrifugation for 5 minutes at 800g and resuspension in ice-cold PBS, the tissue was homogenized with a Polytron (Brinkmann Instruments, Westbury, N.Y.). The final volume of the crude homogenate was adjusted to 2.5 ml, and 250 µl was withdrawn for measurement of protein concentration. A 10-fold concentrate of binding buffer (250 µl) was added. The final composition of the binding buffer was 0.1% BSA, 0.05% bacitracin, 0.01% aprotinin, 0.01 mg/ml phosphoramidon in PBS, pH 7.4. Homogenate (100 µl) was incubated with 50 µl of 125I-ANP (30,000-40,000 cpm) and various concentrations of unlabeled ANP for 2 hours at 4°C. Bound and free ligand were separated by suction filtration and were counted in the same way as the glomerular preparations described above.

In both glomeruli and papillas, nonspecific binding was determined by addition of ≥10^-7 M ANP, and 70–80% of total binding was specific binding. Binding affinity and apparent binding site number were separately determined for each experiment with the computer program LIGAND.14

Cyclic Guanosine Monophosphate Generation Studies

Glomeruli were isolated from clipped and nonclipped kidneys in a manner identical to that described above. After the centrifugation and rinsing steps, the purified glomeruli were sampled for determination of protein concentration and were resuspended in 2.5 ml HBSS/kidney, containing 1 mM isobutyl methyl xanthine (an inhibitor of phosphodiesterases) and 10 mM Hepes, adjusted to pH 7.4. The glomerular suspension was separated into 200-μl aliquots and was incubated for 5 minutes at 37°C in a shaking water bath. Various concentrations of rat ANP 1–28 were then added to the tubes in volumes of 100 or 200 µl. The reaction was stopped after 2 minutes by the addition of 500 µl cold 0.2N HCl; the tubes were vortexed and plunged into ice. After centrifugation for 15 minutes at 4°C and 2,500g, 500 µl of each supernatant was removed by pipetting and was evaporated to dryness in a SpeedVac concentrator (Savant Instruments, Farmingdale, N.Y.). The dried supernatants were stored at 4°C for 2 weeks or less before reconstitution for determination of cGMP concentration by radioimmunoassay.

Analytical Methods

Sodium concentration in plasma and urine was measured by flame photometry. Inulin concentration in plasma and urine was determined by a modified anthrone method.15 Urine volumes were determined gravimetrically. Glomerular filtration rate (GFR) was measured by inulin clearance. All reagents were purchased from Sigma Chemical Co., St. Louis, Mo., except as otherwise noted.

Plasma ANP was measured with a previously characterized radioimmunoassay,7 using 125I-ANP 1–28 from Amersham Corp., Arlington Heights, Ill., and rat ANP 1–28 standards and anti-ANP antiserum from Peninsula Labs. Plasma was extracted on Sep-pak C18 cartridges (Waters Associates, Milford, Mass.) before assay; recovery was 67±5% and reported values are not corrected for recovery. The sensitivity of this assay (ED20) was 3 pg/tube or 30 pg/ml. The within-assay coefficient of variation was 11%; all samples were analyzed in one assay and were done in triplicate.

cGMP was measured in urine and glomerular incubation buffer with kits manufactured by Amersham. The antibody supplied in this kit has been determined by the company to be only 0.02% cross-reactive with cyclic adenosine monophosphate, with lesser cross-reactivities to purines and other nucleotides.

Protein in glomerular suspensions was determined by the method of Lowry et al16 or with a kit manufactured by Bio-Rad, Richmond, Calif., using BSA as the standard.

Paired t tests, analysis of variance, and linear regression and correlation were used as indicated below. Statistical calculations were done with commercially available microcomputer software (STATVIEW 512+, BrainPower, Sepulveda, Calif.).

Results

The results of the blood volume expansion experiments are summarized in Table 1. Mean arterial pressure was relatively constant throughout the experiment, although slight increases were noted after each volume expansion step. The baseline plasma immunoreactive ANP level in the 2K1C hypertensive rats was 107±26 pg/ml.

Over the two volume expansion steps, there was an approximate fourfold rise in plasma immunoreactive ANP (p<0.01), presumably resulting in a similar increase in ANP delivery to both kidneys. There were marked differences in sodium and water excretion between the clipped and nonclipped kidneys, both during control clearances and after blood volume expansion. The nonclipped kidney excreted nearly twice as much water and more than six times as much sodium as the clipped kidney during control periods.
These differences were further accentuated by each blood volume expansion step, which produced marked increases in sodium and water excretion by the nonclipped kidney but minimal or no response on the clipped side. A similar pattern was noted in the values for fractional excretion of sodium, which rose progressively on the nonclipped side after each volume expansion step, but exhibited no increase at all on the clipped side after the first blood volume expansion step, and a slight, nonsignificant increase after the second. On the other hand, mean cGMP excretion was not significantly different at any time between the two kidneys.

GFR per gram kidney weight was not significantly different between clipped and nonclipped kidneys during control clearances. After each volume expansion step, there was an increase in average GFR from each kidney. In the case of the nonclipped kidney, these changes may have been somewhat exaggerated by a dead space error resulting from the sudden large increase in urine flow. The GFR changes after blood volume expansion were not entirely due to dead space errors, however, since the GFR remained above baseline after the urine flow stabilized. In general, the GFR of the nonclipped kidney tended to increase more after blood volume expansion than the GFR of the clipped kidney. Because of substantial variability in this response, differences in GFR between the two kidneys, corrected for kidney weight, did not achieve statistical significance until the last two clearance periods of the experiment.

Figure 1 shows a linear regression of log sodium excretion on simultaneously measured log cGMP excretion for each kidney, determined at various points before and after the two blood volume expansion steps. Although sodium excretion was much

![Graph showing linear regression](image-url)
lower on the clipped side, cGMP excretion per gram kidney weight was not different between the two kidneys (Table 1). On the nonclipped side, there was a highly significant correlation between cGMP and sodium excretion, consistent with the involvement of ANP and its second messenger in the determination of the natriuretic response in this kidney. However, there was no significant correlation between these parameters in the clipped kidney.

The left panel of Figure 2 demonstrates inhibition of iodine-125–labeled ANP binding by addition of various concentrations of unlabeled ANP to isolated glomeruli from the clipped and nonclipped kidneys of a single representative rat. Enough glomeruli could be obtained from one kidney to obtain duplicate measurements of 125I-ANP binding at six to eight different concentrations of unlabeled ANP, in addition to determination of total and nonspecific binding. A Scatchard plot for ANP binding to glomeruli from single clipped and nonclipped kidneys from the same animal is shown in the right panel of Figure 2. The observed straight-line fit after Scatchard transformation of the binding data is compatible with a single class of binding sites in each kidney; this held true in all experiments. Binding parameters were therefore calculated assuming a single-site fit; however, it should be noted that the presence of a second, lower affinity site cannot be confidently excluded with the limited number of binding points that could be examined in each experiment.

Figures 3 and 4 show the results of 10 individual glomerular ANP binding experiments in clipped and nonclipped kidneys. The apparent ANP receptor density (Figure 3) was 500 ± 74 fmol/mg protein (mean ± SEM) in the nonclipped kidney and 575 ± 92 fmol/mg protein in the clipped kidney. The association constant ($K_a$) (Figure 4) was 5.17 ± 0.64 × 10$^9$/mol for the nonclipped kidney and 5.26 ± 0.89 × 10$^9$/mol in the clipped kidney, corresponding to 50% inhibition of binding of 125I-ANP at an unlabeled ANP concentration ($K_d$) of approximately 0.19 nM in each kidney.

Neither receptor density nor binding affinity differed significantly between clipped and nonclipped kidneys, as assessed by paired $t$ tests.

Glomeruli contain two types of ANP receptors, only the minority of which are coupled to guanylate cyclase. It remained possible, therefore, that the subpopulation of guanylate cyclase–coupled receptors was being specifically downregulated in clipped kidneys, without measurably affecting the binding characteristics of the overall receptor population. To address this possibility, ANP-induced cGMP generation by clipped and nonclipped kidney glomeruli in vitro was assessed. Figure 5 summarizes the results of these experiments. A similar monotonic increase in cGMP release with increasing ANP concentration was noted in clipped and nonclipped kidneys. There was no significant difference in ANP dose response between glomeruli from clipped and nonclipped kid-
neies by analysis of variance; if anything, there was a trend toward increased ANP-induced cGMP release relative to baseline by the clipped kidney. The absolute values for cGMP release per milligram glomerular protein were also somewhat higher on the clipped side at baseline and at each ANP dose level, although again the difference was not statistically significant (data not shown).

The results of four binding experiments with pooled crude inner medullary homogenates are shown in Table 2, and the displacement curves and Scatchard plots for a representative experiment are shown in Figure 6. As in the glomeruli, the data in all experiments were consistent with, but not definite proof of, the existence of a single class of receptors.

Binding affinity was not different between clipped and nonclipped kidneys, and was similar (approximately twofold lower) to that measured in glomeruli. Apparent binding site density was higher in the clipped than in the nonclipped kidney papillas in four of four experiments, whether expressed as femtmoles per milligram protein or femtmoles per papilla. However, the overall difference did not achieve statistical significance.

**Discussion**

The results indicate that renal glomerular ANP receptors in the clipped kidney, which has a blunted response to ANP, are equally numerous, bind ANP with equal avidity, and produce at least as much cGMP in the presence of ANP as those in the ANP-responsive nonclipped kidney. Similarly, the density of papillary ANP receptors was at least as high in the clipped as in the nonclipped kidney. In the nonclipped kidney, there was a good correlation between urinary cGMP and the natriuretic response to blood volume expansion, supporting a close link between ANP secretion and renal response. However, this correlation was absent in the clipped kidney. Taken together, these findings indicate that the pronounced end-organ resistance to the actions of ANP in the clipped kidney is due to mechanisms other than downregulation of receptor number or function in this kidney.

These findings contrast with those of Gauquelin et al. who found fourfold fewer ANP receptors in clipped kidney glomerular membranes than in the nonclipped kidney. These investigators found the changes in receptor binding occurred 3 weeks after clipping, which is the same time period that we examined, and also used a 0.2 mm clip, the same size that we used. The reasons for the disparity in our results is not clear, although there were some differences in experimental animals and in radioreceptor assay technique between the two studies. Gauquelin et al. used pooled, stored glomerular membranes from 10 experimental animals, whereas we per-
formed 10 individual experiments in freshly isolated glomeruli from single animals. The rats described by Gauquelin et al.\textsuperscript{10} were larger when clipped (200–250 g) than ours (100–125 g) and had mean arterial pressures of 143 mm Hg. The rats in the present study which underwent clearance experiments had more severe hypertension at 3 weeks after clipping (mean arterial pressure 170 mm Hg).

Gauquelin et al.\textsuperscript{10} also examined the time course of receptor number regulation after clipping and found that the difference in receptor density between glomeruli from the two kidneys was transient. We have not examined other time points in the development of 2K1C hypertension with regard to receptor characteristics. However, our studies do demonstrate that resistance to the natriuretic effect of blood volume expansion in the clipped kidney occurs at the same time that no decrease in ANP receptor number or function in this kidney relative to the responsive nonclipped kidney can be demonstrated. On the basis of their results, Gauquelin et al.\textsuperscript{10} proposed that changes in ANP receptors contribute to the impaired sodium excretion ability of the clipped kidney. The results of the present study contradict this hypothesis.

Another study from this group found that a decreased number of ANP receptors in pooled glomerular membranes from clipped kidneys was seen in both saralasin-sensitive, presumably renin-dependent, 2K1C rats and in saralasin-resistant rats.\textsuperscript{11} It is well established that dependency of hypertension on the renin-angiotensin system in 2K1C rats varies, particularly with the time after clipping.\textsuperscript{17} Angiotensin II can apparently regulate ANP binding site density in some cells both in vivo and in cell culture systems.\textsuperscript{18,19} We did not measure plasma and tissue renin or angiotensin II concentrations in the present study. If our animals happened to have relatively saralasin-resistant hypertension, and renal angiotensin II levels were not grossly elevated then, according to the findings of Garcia et al.\textsuperscript{11} the differences in glomerular ANP receptors may not have been as marked as if the opposite were true. Nevertheless, a marked disparity in natriuretic responsiveness between the clipped and nonclipped kidneys was still present in this study, and no decrease in ANP receptor density in the clipped kidney could be confirmed.

The present study was therefore unable to determine the mechanisms of impaired natriuresis in the clipped kidney. A number of potential antinatriuretic mechanisms that were not assessed or manipulated are likely to be operative in the clipped kidney, such as angiotensin II,\textsuperscript{20} renal sympathetic nerve activity,\textsuperscript{21} and vasoconstrictor arachidonate metabolites.\textsuperscript{22} The magnitude of ANP-induced sodium excretion is quite sensitive to renal perfusion pressure.\textsuperscript{23} Lowitz et al.\textsuperscript{24} found that the perfusion pressure behind a 0.2 mm clip in 2K1C rats with established hypertension is close to "normotensive."\textsuperscript{24} In their study, the mean arterial pressure was 175 mm Hg in the clipped animals, 121 mm Hg in normal animals, and 119 mm Hg behind the clip. Since normal kidneys at normal perfusion pressure are able to respond to

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{Line graph shows competitive inhibition of binding of iodine-125-labeled atrial natriuretic peptide (I\textsuperscript{125}I-ANP) by unlabeled ANP in papillary homogenates prepared from four clipped and four nonclipped kidneys in left panel. Nonspecific binding has been subtracted. Scatchard transformation of the data is shown in right panel. b/f, bound/free.}
\end{figure}
ANP, it is unlikely that the perfusion pressure to the clipped kidney entirely accounts for its markedly blunted response to blood volume expansion. The clipped kidney in the present clearance studies had a normal baseline GFR, indicating that the perfusion pressure to this kidney under the experimental conditions used was within the autoregulatory range and that lack of a natriuretic response was not due to renal failure, and suggesting that inability to alter tubular sodium and water reabsorption after blood volume expansion may be important in producing resistance to the effects of this maneuver in the clipped kidney.

The GFR appeared to increase more after blood volume expansion in the nonclipped kidney than in the clipped kidney. Although this difference was not initially statistically significant, even a small increase in GFR, if unbuffered by corresponding alterations in tubular transport, would result in marked natriuresis. Since ANP can increase GFR, some authors think that the resulting increase in filtered sodium and water is the major reason for the diuretic and natriuretic response. It may be, therefore, that the relatively blunted rise in GFR in the clipped kidney after blood volume expansion could have contributed to the observed lack of natriuretic response. It is also possible that the increase in GFR in the nonclipped kidney was due in part to ANP release into the circulation after blood volume expansion. If ANP did contribute to the GFR increase in the nonclipped kidney, then it appears that the clipped kidney was relatively resistant to this ANP effect as well.

In summary, it remains uncertain why the clipped kidney exhibits marked blunting of the sodium and water excretory response to ANP infusion or blood volume expansion. The present study provides evidence that numerical or functional downregulation of renal ANP receptors is not a necessary contributing factor and that other mechanisms for resistance to ANP action are operative in the clipped kidney.

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References


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