Papillary Collecting Tubule Responsiveness to Atrial Natriuretic Factor in Dahl Rats

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SUMMARY There is evidence that atrial natriuretic factor (ANF) has an action in the inner medullary collecting duct. In addition, the prehypertensive Dahl salt-sensitive (S) rat has an intrinsic tendency toward less natriuresis than the Dahl salt-resistant (R) rat has when challenged with ANF. To test the hypothesis that renal papillary collecting tubule cells from prehypertensive S rats might be genetically less responsive to ANF, S and R cells were grown in culture and studied for responsiveness to ANF by measurement of cyclic nucleotide responses. There was a concentration-dependent effect of ANF on renal papillary collecting tubule cell synthesis of intracellular cyclic guanosine 3',5'-monophosphate (cGMP) in both strains. However, the S cells were hyporesponsive compared with the R cells (p<0.002, by analysis of variance). Likewise, in response to Na nitroprusside, the S cells were hyporesponsive compared with the R cells as measured by intracellular cGMP accumulation (p<0.03, by analysis of variance). Arginine vasopressin stimulated intracellular cAMP equally in both strains. Also, ANF equally enhanced intracellular cGMP in glomerular mesangial cells from S and R rats, indicating possible specificity of the reduced responsiveness to ANF to the distal nephron of S rats. Plasma ANF levels had a slight tendency to be higher in prehypertensive S rats than in R rats (p = 0.088, by t test). These results suggest that the papillary collecting duct of Dahl S and R rats may differ in guanylate cyclase activity. This difference may partially explain the impaired natriuretic responses of S rats and could represent a factor contributing to the development of salt-sensitive hypertension. (Hypertension 10: 107-114, 1987)

KEY WORDS • atrial natriuretic factor • papillary collecting tubule • Dahl rats • cyclic GMP • cell culture

THE discovery of atrial natriuretic factor (ANF) has stimulated interest in the mechanism of action of this hormone.1 A variety of studies have implicated the glomerulus as a site of action for ANF, suggesting that renal hemodynamic effects contribute to the natriuretic actions of the hormone.2-6 The present study used biochemical techniques to focus on the controversial issue of whether ANF acts directly on the renal tubule. Previous work has implicated the distal nephron as a site of action of ANF.7-10 ANF stimulates cyclic guanosine 3',5'-monophosphate (cGMP) synthesis in cultured epithelial cells derived from the papillary collecting duct.9,10 In addition, specific ANF receptor binding has been demonstrated in preparations of inner medullary collecting tubule fragments.11-13 More recently, evidence has accumulated suggesting a physiological role of ANF may be to inhibit NaCl transport at the level of the inner medullary collecting duct.14,15

Intravenous injection of atrial extracts in prehypertensive Dahl salt-sensitive (S) rats induces less natriuresis and diuresis than in Dahl salt-resistant (R) rats.16,17 This finding has been demonstrated in both the Brookhaven16 and Toledo17 strains of Dahl rats. In both strains, the natriuresis in the S rats was approximately 50% of that seen in the R rats. In addition, Tobian et al.18 have reported that isolated blood-perfused kidneys obtained from prehypertensive S rats required a higher perfusion pressure to excrete an amount of sodium equivalent to that excreted by R rats. Recently, Roman19 confirmed this finding in an in vivo model that demonstrated the diuretic response of prehypertensive S rats to acute elevations in arterial pressure was less than half that seen in R rats.
Thus, there is evidence to support the concepts that 1) ANF has an action in the inner medullary collecting duct; 2) the prehypertensive Dahl S rat has an intrinsic tendency toward less natriuresis than the Dahl R rat when challenged with ANF or increasing renal perfusion pressure. We therefore hypothesized that the inner medullary collecting duct of the prehypertensive S rat, as compared with that of the R rat, might be genetically less responsive to the natriuretic effects of ANF. To this end, renal papillary collecting tubule (RPCT) cells from S and R rats were cultured in parallel and studied for ANF responsiveness by biochemical measurement of cyclic nucleotides. The findings of these experiments may point to a factor contributing to the development of salt-sensitive hypertension in the Dahl rat.

**Materials and Methods**

**Atrial Natriuretic Factor**

The synthetic rat atrial natriuretic peptides atriopeptin I (AP I; 21 amino acids) and atriopeptin III (AP III; 24 amino acids) were purchased from Bachem (Torrance, CA, USA). Synthetic ANF (8-33; 26 amino acids) was a gift from Dr. Edward Blaine (Merck Sharp & Dohme, West Point, PA, USA). This peptide, as well as AP III, have been shown to have properties similar to those of the purified low molecular weight peptide, whereas AP I is substantially less natriuretic and vasorelaxant.20,21 The synthetic peptides were dissolved in phosphate-buffered saline, with 0.2% (wt/vol) fatty acid-free albumin, bovine fraction V (Calbiochem-Behring, La Jolla, CA, USA) added to prevent possible adhesion of the peptide to plastic. Stock solutions were frozen at -40°C and diluted to the proper concentration at the time of experimentation.

**Animals**

Male Sprague-Dawley rats (Zivic-Miller, Allison Park, PA, USA), weighing 125 to 150 g and fed normal rat chow, were used in preliminary experiments designed to study the time-response and dose-response effects of the ANF peptides on cGMP and cyclic adenosine 3',5'-monophosphate (cAMP) accumulation in RPCT cells in culture. Dahl S and R rats of the Toledo strain (SS/Jr and SR/Jr, respectively) were used in all other experiments. Breeders were a gift from Dr. John Rapp (Toledo, OH, USA). A colony was then propagated at our institution. Weanling SS/Jr and SR/Jr of both sexes were placed on a low salt diet containing 0.3% NaCl (ICN Biochemicals, Irvine, CA, USA) until the age of 5 to 6 weeks. At that time, the prehypertensive SS/Jr and SR/Jr were killed for cell culture experiments. In another group of rats, weanling SS/Jr and SR/Jr of both sexes were fed normal rat chow until 6 weeks of age. At that point, 24-hour urine samples were collected in metabolic cages for cyclic nucleotide determinations. These rats were killed by rapid decapitation, and trunk blood was collected into ice-cold tubes containing 3 mg of potassium EDTA (Becton Dickinson, Rutherford, NJ, USA) and 1000 kallikrein-inhibiting units (KIU) of aprotinin (Sigma Chemical, St. Louis, MO, USA). Plasma was then processed for ANF levels as will be described.

**Cell Cultures**

RPCT cells were isolated using previously described techniques.24,25 Kidneys were removed from ether-anesthetized rats, and the papillae were dissected and finely minced. The tissue was incubated for 2 hours at 37°C in phosphate-buffered saline containing collagenase (CLS II; Worthington Biochemical, Freehold, NJ, USA) at a concentration of 1 mg/ml. Hypotonic lysis of cells other than RPCT cells was accomplished by the addition of distilled water, yielding a final osmolality of 120 mosm/kg. After a 10% albumin density centrifugation, the intact RPCT cells were suspended in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) and Ham’s F-12 medium (Gibco) supplemented with 10% fetal bovine serum (K.C. Biological, Lenexa, KS, USA) to facilitate attachment to polystyrene culture dishes (Costar, Cambridge, MA, USA). After 24 hours, the media were changed to a serum-free, fully defined K-1 media that favors epithelial cell growth.24 Media were changed daily until the cells reached confluence at 3 to 4 days. Experiments were performed on the primary cultures.

Glomerular mesangial cells were started from isolated glomeruli using the method of Lovett et al.25 Mesangial cells were grown in polystyrene culture dishes in RPMI 1640 media (K.C. Biological) containing 17% fetal bovine serum at 37°C in 5% CO2, 95% air. Primary cultures were incubated for approximately 1 week until the cells approached confluence. The cells were subcultured, and experiments were performed on cells approaching confluence from Passages 2 to 4.

**Cell Culture Cyclic Nucleotide Responses**

Experimental incubations were performed on RPCT and mesangial cell monolayers. Triplicate determinations of cGMP and cAMP were made under each experimental condition in each cell line. The cells were washed twice in Eagle’s minimal essential media (M.A. Bioproducts, Walkersville, MD, USA) with 25 mM HEPES. Incubations were carried out in room air at 37°C in the same media in the presence of 0.1 mM 3-isobutyl-1-methylxanthine (Sigma). Dose-response data for the effect of the synthetic ANF peptides, Na nitroprusside (Sigma), and arginine vasopressin (Calbiochem-Behring) on intracellular cGMP and cAMP were generated by the addition of the agonist at time zero, followed by a 3-minute incubation that was ended by the removal of the experimental media, followed by the addition of 0.1 N HCl, which extracts intracellular cyclic nucleotides from cell monolayers.26 The extract was frozen at -40°C and saved for radioimmunoassay (RIA).
Assays

Intracellular and urinary cyclic nucleotides were measured by RIA. Urinary cyclic nucleotides were measured unextracted, since at least a 5000-fold dilution of urine was required before assay. RIA for cGMP was done with 125I-tyrosine methyl ester cGMP (New England Nuclear, Boston, MA, USA), and rabbit antisera to cGMP (Kew Scientific, Columbus, OH, USA). The cross-reactivity of the cGMP antisera with cAMP was 0.001%, and for guanosine 5'-monophosphate (GMP), guanosine 5'-diphosphate (GDP), guanosine 5'-triphosphate (GTP), adenosine 5'-monophosphate (AMP), and adenosine 5'-triphosphate (ATP) it was less than 0.001%. RIA for cAMP was done with 125I-tyrosine methyl ester cAMP prepared by the chloramine T method followed by thin-layer chromatography purification. Goat antisera to cAMP (Research Products International, Mount Prospect, IL, USA) was used, which had a cross-reactivity of less than 0.001% with AMP, adenosine 5'-diphosphate (ADP), ATP, GMP, GTP, and cGMP. For both cGMP and cAMP RIA's, samples were done in duplicate or triplicate after acetylation. There was no effect of the various agonists, 0.1 N HCl, 0.1 mM 3-isobutyl-1-methylxanthine, or incubation media on ligand-antiserum binding.

Cellular protein was measured in each culture dish after an overnight digestion with 1 N NaOH by the Lowry method using bovine serum albumin as standard. In all cases, protein concentrations of cultures from SS/Jr and SR/Jr were similar. Therefore, it is quite unlikely that intrinsic differences in plating efficiency or cell growth could account for any differences noted.

Plasma ANF was measured by RIA after extraction as described by Burnett et al. As previously noted, trunk blood from decapitated SS/Jr and SR/Jr was collected into iced tubes containing 3 mg of EDTA and 1000 KIU of aprotinin. Plasma was immediately separated from whole blood and frozen at -40°C. Plasma was extracted by application to Sep-Pak C cartridges (Waters Associates, Milford, MA, USA), primed with methanol and water. ANF was eluted with a solution containing 75% methanol and 1% trifluoroacetic acid. This eluate was dried under nitrogen and reconstituted in buffer for RIA. The recovery of ANF through the extraction procedure was 78 ± 4 (SEM)% (n = 5), determined by addition of synthetic ANF to plasma. The RIA for ANF was done with 125I-α-atrial natriuretic polypeptide (αANP) (Peninsula Laboratories, Belmont, CA, USA), and rabbit anti-αANP antisera (Peninsula). A standard curve was constructed by serial dilution of rat αANP (28 amino acids; Bachem, Torrance, CA, USA) from 500 to 2.5 pg/100 μl. The nonequilibrium RIA uses a second antibody to separate free and bound fractions of αANP. Total binding was 39%, and 50% displacement of 125I-αANP occurred with 26 pg of rat αANP.

Results

RPCT cells had morphological characteristics of typical inner medullary collecting duct epithelia, including rudimentary luminal microvilli, tight intercellular junctions, large nuclei near the basolateral surface of the cells, and a columnar appearance. Phase-contrast microscopy demonstrated a cobblestone appearance to the monolayer, with individual polygonal cells. Small fragments of inner medullary collecting tubules were seen along with an occasional hemicyst.

Time and Dose Response

Preliminary experiments were done on RPCT cells grown from Sprague-Dawley rats fed normal rat chow. Figure 1 shows that ANF (8–33), 3.2 × 10⁻⁷ M, substantially increased intracellular and extracellular cGMP. The peak intracellular response occurred within 3 minutes and then slowly decreased but remained elevated at 45 minutes. Extracellular cGMP increased progressively over 45 minutes. Figure 2 demonstrates that both AP I and AP III caused a concentration-dependent increase of intracellular cGMP accumulation. The differential effects were marked, as AP III had a lower threshold and a greater effect on intracellular cGMP than did AP I, thus paralleling the comparatively greater natriuretic and diuretic effect of AP III. Similarly, ANF (8–33), two amino acids longer than AP III, was more potent than AP III. Values of intracellular cGMP were 4.6 ± 0.6, 14.0 ± 0.6, and 29.1 ± 2.6 fmol/μg protein per 3-minute incubation when the cells were incubated with 3.2 × 10⁻⁸, 10⁻⁷, and 10⁻⁶ M ANF (8–33), respectively. (Values represent means ± SEM of replicate wells from two to six separate cell lines.) Considering these differential effects, we felt that the measurement of cGMP could be useful in the RPCT cell as an index of ANF responsiveness. None of the ANF peptides had any effect on intracellular cAMP in RPCT cells. In the presence of 0.1 mM 3-isobutyl-1-methylxanthine, basal levels of cAMP were 25.4 ± 2.8 fmol/μg protein per 3-minute incubation as compared with 31.8 ± 1.4, 27.6 ± 3.3, and 24.3 ± 1.2 fmol/μg protein per 3-minute incubation when the cells were exposed to 1 × 10⁻³, 10⁻⁴, and 1 × 10⁻⁵ M ANF (8–33), respectively. (Values represent means ± SEM of triplicate wells from two separate cell lines.)
FIGURE 2. Dose-response effect of atriopeptin I (AP I) and III (AP III) on intracellular cGMP accumulation during 3-minute incubations in renal papillary collecting tubule cells grown from Sprague-Dawley rats fed normal rat chow. All incubations were done in the presence of 0.1 mM 3-isobutyl-1-methylxanthine. Data points represent the means ± SEM of triplicate wells from two to six separate cell lines. By analysis of variance, there was a significant difference for the combined effects of dose and ANF peptide (p < 0.01). Single (p < 0.03) and double (p = 0.07) asterisks indicate significant difference compared with the same dose of AP I (by independent t test).

and 10⁻⁶ M ANF, respectively. (Values represent means ± SEM of replicate wells from three separate cell lines.)

Blood Pressure Responses in Dahl Rats

All remaining data were obtained from SS/Jr and SR/Jr. As can be seen in Figure 3, neither SS/Jr nor SR/Jr were hypertensive while ingesting 0.3% NaCl, although the SS/Jr had a slightly higher blood pressure than did the SR/Jr while on 0.3% NaCl. With the institution of a high salt diet containing 4.0% NaCl, the SR/Jr remained normotensive at 120 mm Hg, while SS/Jr rapidly became markedly hypertensive with systolic blood pressure increasing from 130 to 200 mm Hg. Because we were interested in studying the onset phase of salt-sensitive hypertension, RPCT cell cultures were done on SR/Jr and prehypertensive SS/Jr at 5 to 6 weeks of age on a 0.3% NaCl diet.

Cyclic Nucleotide Responses in Dahl Rats

Figure 4 shows the data from seven separate RPCT cell lines grown from 5- to 6-week-old SR/Jr and prehypertensive SS/Jr ingesting 0.3% NaCl diets. Cells from SS/Jr and SR/Jr demonstrated a concentration-dependent effect of AP III on intracellular cGMP, but at each concentration above threshold, the cells from SS/Jr were hyporesponsive compared with cells from SR/Jr. This differential effect reached statistical significance at the higher concentrations tested, where the response in the cells from SS/Jr was 25 to 30% less than that in the cells from SR/Jr.

To test whether this differential finding was specific to the hormone AP III, we tested other agonists that have effects on cyclic nucleotides. The actions of Na nitroprusside, which stimulates guanylate cyclase independently of receptor-mediated mechanisms, are shown in Figure 5. Cells from both SS/Jr and SR/Jr responded to nitroprusside in a concentration-dependent manner with increases in intracellular cGMP accumulation. However, again, the cells from SS/Jr were hyporesponsive compared with the cells from SR/Jr.
ANF AND THE COLLECTING DUCT IN DAHL RATS/Appel and Dunn

111

FIGURE 5. Dose-response effect of Na nitroprusside on intracellular cGMP accumulation during 3-minute incubations in renal papillary collecting tubule cells grown from 5- to 6-week-old SR/Jr (R) and prehypertensive SS/Jr (S) fed a low salt diet (0.3% NaCl). All incubations were done in the presence of 0.1 mM 3-isobutyl-1-methylxanthine. Data points represent the means ± SEM of triplicate wells in four separate cell lines. By analysis of variance, there was a significant difference for the combined effects of dose and strain (p<0.03). Asterisk indicates significant difference compared with same-dose values for SS/Jr (p<0.01, by independent t test).

Are cells from SS/Jr somehow damaged and therefore hyporesponsive (compared with cells from SR/Jr) to the effect of these agonists? This was not the case, as shown in Figure 6, where the effects of arginine vasopressin on cAMP accumulation are depicted. Cells from both SS/Jr and SR/Jr responded to vasopressin in a concentration-dependent manner with increases in intracellular cAMP. However, there was no difference in synthetic capabilities between cells from the SS/Jr and SR/Jr.

Next, we wondered whether diminished cGMP responsiveness is a finding specific to the distal tubule or whether it represents a genetic strain difference that manifests itself throughout the nephron. To answer this question, we studied the effect of AP III on cGMP accumulation in glomerular mesangial cells grown from SS/Jr and SR/Jr. As shown in Figure 7, whereas both strains responded to AP III, there were no differences between the mesangial cells from SS/Jr and SR/Jr. Thus, there is some specificity of the differential response noted in the RPCT cells.

Plasma ANF and Urinary Cyclic Nucleotides in Dahl Rats

Table 1 summarizes data on plasma ANF and urinary cyclic nucleotides obtained from 6-week-old SR/Jr and prehypertensive SS/Jr fed normal rat chow (0.7% NaCl). There was a tendency for plasma ANF levels to be higher in the prehypertensive SS/Jr compared with the SR/Jr; however, this tendency did not reach statistical significance. Cyclic nucleotide excretion rates were similar in the two strains.

Discussion

It has been known for some time that the kidney is important in the development of salt-sensitive hypertension. Dahl and Heine\(^2^9\) demonstrated that transplantation of a kidney from an S rat to an R rat transfers salt sensitivity to the R rat. Tobian et al.\(^1^8\) have shown that, in the isolated perfused kidney, equal perfusion pres-
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to Na nitroprusside in the cells from SS/Jr was approximately 30% at higher concentrations. Likewise, the response to ANF was less in young, prehypertensive animals, most of which were SR/Jr. The difference was quantitatively similar to that in RPCT cells from SR/Jr. The dose response to AP HI in Sprague–Dawley rats (see Figure 2) was quantitatively similar to that in SR/Jr (see Figure 4) and somewhat less than that seen in the SR/Jr (see Figure 4). This finding could suggest that SR/Jr are more sensitive than normal rats and that cGMP accumulation has nothing to do with salt sensitivity. However, we believe that the proper control for the S rat is the R rat, and not the Sprague–Dawley rat. It is not clear whether similar levels of cGMP in the papillary collecting duct of SS/Jr and Sprague–Dawley rats would result in similar functional responses. It is more likely that differences in cGMP between SS/Jr and SR/Jr translate to a differential functional response.

Do these biochemical findings contribute to the pathophysiology of salt-sensitive hypertension? We believe the answer may be affirmative for several reasons. There is increasing evidence that the inner medullary collecting duct is a site of action for ANF. In addition to the clear biochemical interaction shown in the present study, ANF receptor binding has been demonstrated in preparations of inner medullary collecting tubules.11–13 Two recent studies showed a physiological interaction of ANF at this site. With the use of a microcatheterization technique, Sonnenberg et al.14 reported that infusion of ANF in the rat reduced fractional sodium reabsorption in the inner medullary collecting duct. Zeidel et al.15 showed that ANF diminished oxygen consumption in suspensions of rabbit inner medullary collecting duct in a manner compatible with inhibition of sodium entry. The evidence that cGMP plays a role in nephron transport processes has been quite limited; however, work by Zeidel et al.13 has shown that 8-bromo cGMP entirely mimicked the ANF inhibition of Na+ transport-dependent oxygen consumption in suspensions of rabbit inner medullary collecting duct. Thus, it appears that our measurement of cGMP in RPCT cells may have pathophysiological relevance, especially since the findings were specific for cGMP responsiveness in epithelia from the distal nephron.

The reduction in cGMP accumulation in SS/Jr in

<table>
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<th>Variable</th>
<th>SS/Jr</th>
<th>SR/Jr</th>
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<tr>
<td>Plasma ANF (pg/ml)</td>
<td>250 ± 17 (10)</td>
<td>213 ± 11* (9)</td>
</tr>
<tr>
<td>Urinary cGMP (nmol/24 hr)</td>
<td>56.9 ± 3.2 (8)</td>
<td>61.0 ± 3.4 (9)</td>
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<tr>
<td>Urinary cAMP (nmol/24 hr)</td>
<td>121.2 ± 7.2 (8)</td>
<td>126.9 ± 9.2 (9)</td>
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response to both ANF and nitroprusside argues against a deficit in ANF receptor number or affinity in the S strain as a single defect. There may be a defect in solubility as well as particulate guanylate cyclase activity. The data do not rule out the possibility that a guanylate cyclase defect may coexist with a problem at the level of the ANF receptor in the S strain. Is the defect a genetic one, or is it acquired? The cell culture conditions employed in this study would favor a genetically determined defect since acquired defects tend to disappear with cell replication in culture. On the other hand, a genetic defect would likely be expressed in mesangial as well as RPCT cells. Thus, the issue is unresolved.

We measured plasma ANF to look for an elevation of the hormone, as might be expected in the presence of target hyperresponsiveness. Although plasma ANF was numerically greater in the young, prehypertensive SS/Jr, the differences were small and perhaps not meaningful. Plasma ANF is reported to be higher in older, salt-fed hypertensive S rats compared with R rats.34 No difference in urinary cGMP excretion was found in this study. It may be necessary to infuse ANF in S and R rats to bring out differences in cGMP excretion.

In conclusion, we have demonstrated a relative insensitivity in the papillary collecting duct of the prehypertensive SS/Jr to the actions of ANF to increase intracellular cGMP. Since even small differences in responsiveness in the most distal portion of the nephron could markedly change the reabsorption of sodium, these results suggest a partial explanation for the lesser natriuresis induced by ANF in the prehypertensive S rat as compared with R rats. This alteration could markedly change the reabsorption of sodium. The effect of ANF on intracellular cGMP was lost in mesangial as well as RPCT cells. Thus, the issue is unresolved.

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_Hypertension_. 1987;10:107-114
doi: 10.1161/01.HYP.10.1.107

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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