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Role of Atrial Natriuretic Factor in Regulation of Blood Pressure in Normotensive Rats Having Reduced Renal Mass[1] (42810)

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Abstract. Experiments were carried out in normotensive, saline-drinking, 60% reduced renal mass rats to determine the effect of an *in vivo* blockade of endogenous atrial natriuretic factor (ANF) on blood pressure. We used a 60% reduction in renal mass because blood pressure in these normotensive animals is extremely sensitive to any slight further reduction of renal excretory function. Six weeks following the reduction of renal mass and documentation of normotension, rats were injected intraperitoneally twice daily for 12 days with ANF antibody prepared against the C-terminal heptapeptide of AP III conjugated to bovine thyroglobulin. Control rats similarly prepared, received normal rabbit serum (NRS). Blood pressure progressively increased in rats receiving the antibody, and its withdrawal returned blood pressure to control levels within 4–5 days. Serum from either normal rabbits or rabbits immunized with bovine thyroglobulin or peptides unrelated to ANF had no effect on blood pressure in the control animals. These experiments show that in the normotensive saline-drinking rat with reduced renal mass, an antibody to AP III raises blood pressure. This suggests that ANF here is acting to prevent the rise in blood pressure.

It is well established that secretory granules of atrial cardiocytes contain potent diuretic and natriuretic activities which are often referred to as atrial natriuretic factors (ANF) (1–4). More recently, these activities have been identified with several structurally related, atrial natriuretic peptides (ANPs), which have been isolated, purified, and sequenced (5–11). Some of these active peptides having Phe–Arg or Phe–Arg–Tyr residues on the carboxyl terminal also possess vasorelaxant activity when examined *in vitro* (12–14). However, *in vivo* a decrease in total peripheral resistance is not consistently observed following intravenous administration of ANPs. Some investigators report no effect of the atrial peptides on blood pressure in normotensive experimental animals (4, 15–17), whereas others report a mild or transient hypotensive effect (1, 18, 19). In contrast, intravenous infusion of extracted atrial ANF or synthetic atriopeptins consistently decreases blood pressure in animals with certain types of experimental hypertension (15, 16, 18). Furthermore, both decreased and increased tissue levels of ANF have been reported in some experimental models of hypertension (20–22), while plasma levels of ANF are elevated (23, 24) or normal (25). In addition, most studies have examined only the short-term effects of intravenously administered ANF. Thus the role of ANF in regulation of blood pressure is not clear.

One possibility is that, through its natriuretic and vasodilator actions, ANF acts to compensate the blood pressure under conditions characterized by salt and water retention. We reasoned that this might be demonstrated if the actions of ANF were blocked in an animal with a compromised ability to control blood pressure. We, therefore, used an ANF antibody, prepared against the C-terminal heptapeptide of AP III, to block the *in vivo* action of endogenous ANF in normotensive saline-drinking, rats having 60% reduced renal mass.

Materials and Methods. *Preparation of reduced renal mass rats.* Male Wistar rats (250–280 g) with documented normotension underwent a 60, 65, or 70% (±0.5%) surgical

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reduction in renal mass under ether anesthesia, as previously described (26). Briefly, through a midabdominal incision, the right kidney (50% of total renal mass) and poles of the left kidney (10, 15, or 20% of total renal mass) were removed. These calculations are based on the assumption that the weight of both kidneys is equal. The latter was achieved by encircling the poles with a loop of No. 4.0 silk suture and then tightening the loop. This method both cuts the tissue and ties off the arteries and veins in the excised area. Following recovery from surgery, all animals consumed a low-sodium (0.02% Na, BioServices, Inc., Frenchtown, NJ) diet and drank 1% NaCl solution *ad libitum*. Systolic blood pressure (tail plethysmography, Natume-KN209, Tokyo, Japan) and body weight were monitored weekly.

Preparation of ANF antibody. Six antisera were developed in New Zealand white rabbits against the C-terminal heptapeptide of AP III (Lys–Cys–Asn–Ser–Phe–Arg–Tyr) (Peninsula Laboratories Inc., Belmont, CA) as previously described (27). In brief, the peptide (10 mg) was conjugated to bovine thyroglobulin (100 mg; dissolved together in 1 ml H_2O) with the dropwise additon of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (500 mg in 1 ml H_2O). Following dialysis, the conjugates were emulsified with Freund's complete and incomplete adjuvants (2:1:1) and administered in multiple intradermal injections. Booster injections were given at 4- to 6-week intervals and blood samples were collected periodically for antibody analysis. Radiolabeled AP III (2 μg) was prepared by the standard chloramine-T iodination procedure and purified by reverse-phase chromatography on Sep-Pak cartridges (Waters Associates, Inc., Milford, MA). The antiserum used here, X-80, was determined to have the highest titer and sensitivity for AP III. At a final dilution of 1:50,000 it binds 30–40% of the radiolabeled AP III (20,000 cpm) and is sensitive to less than 10 fmole of AP III standard. The C-terminal tyrosine of AP III is an absolute requirement for antibody recognition; accordingly AP I, AP II, and other C-terminal shortened forms of AP III are not detected. The antibody also readily detects preproANF, apparently on an equimolar basis with AP III. In the radioimmunoassay, acetic acid extracts of rat atria and unextracted plasma produced a dose-dependent inhibition curve which paralleled synthetic AP III standards.

Administration of ANF antibody to normotensive 60% reduced renal mass rats. Rats with documented normotension for 5 weeks after a 60% reduction in renal mass and consuming a sodium-free diet and drinking 1% saline were randomly divided into experimental and control groups. The experimental rats were injected intraperitoneally with 0.5 ml ANF antibody twice daily for 12 days. This dose and schedule were selected on the basis of our findings resulting from immunoneutralization studies involving other antisera. Following a single intraperitoneal injection of rabbit antiserum into rats, concentrations of circulating antibodies increase progressively for 3–4 hr, remain maximally elevated for 4–8 hr, and return toward control values by 12 hr postinjection. Antibody titers are usually still detectable by 24 hr after a single injection. Additionally, preliminary experiments showed that the injection of this dose of ANF antiserum tended to raise blood pressure in 2–4 days in 60% reduced renal mass rats for 12 days. The control rats were treated similarly except that they received normal rabbit serum. To determine the effect of treatment on serum composition, 6 ml of aortic blood was collected from X-80 and normal rabbit serum-injected, 60% reduced renal mass rats at the end of treatment period. The blood was allowed to clot in polypropylene tubes at room temperature and spun for 10 min in a refrigerated centrifuge (Sorvall RC-5; SM-24 motor), and the serum was separated for composition analysis.

Sodium and potassium concentrations were measured by flame photometry (Beckman Klina Flame), chloride by chloride titration (Radiometer, Copenhagen, CMT 10 chloride titration), osmolality by freezing-point depression osmometer (Advanced Instruments, Inc., Model 3D II), creatinine by the spectrophotometric (Beckman Model 2 spectrophotometer) method of Bonsnes and Tausky (28), blood urea nitrogen (BUN) by the method of Davidson and Wells (29), protein by the biuret method, and Ca^{2+} and

Mg^{2+} by atomic absorption spectrophotometry (Perkin–Elmer 603).

In a second series of animals, blood pressures were monitored daily during the 12-day X-80 treatment period and then for 12 days following cessation of treatment.

A third series of experiments was conducted to demonstrate the specificity of the blood pressure response to anti-ANF. We injected 60% reduced renal mass rats consuming a sodium-free diet and drinking 1% saline with control hyperimmune serum (0.5 ml, twice daily) obtained from rabbits treated in a manner identical to that of X-80, except that these sera had no anti-ANF antibody titer. Other 60% reduced renal mass rats were injected with antisera developed against peptides which are unrelated to ANF, namely, anti-Ac-β-endorphin (anti-Ac-β-End) or anti-cholecystokinin (anti-CCK).

Statistical analysis of data. The data were statistically analyzed using analysis of variance of repeated measures (TSRM) followed by Duncan's multiple range test. A P value below 0.05 was considered significant. Student's t test was used to statistically analyze body weights and serum constituents of rats treated with ANF antibody or normal rabbit serum. The paired t test was used to compare body weights of rats before and after the treatment. Values of $P < 0.05$ were considered significantly different.

Results. Figure 1 shows the effects of reductions in renal mass on systolic blood pressure in rats. As expected, systolic blood pressure increased progressively from the first through the fifth week following a 70% reduction in renal mass and was significantly greater than the systolic blood pressure of these rats prior to surgery. By the fifth week it had reached 157.6 ± 1.0 mm Hg, an increase of 38% over the control level. Similarly, systolic blood pressure increased significantly and progressively from the first through the fifth week following 65% reduction in renal mass. However, the increase in blood pressure in the 65% reduced renal mass rats was less than the increase in blood pressure in rats with a 70% reduction in renal mass. By the end of the fith week it had reached only 148.4 ± 3.6 mm Hg, an increase of 23.9% over the control level. In contrast, following the 60% reduction in renal mass the animals remained normotensive. The systolic blood pressure in these animals increased by only 4.9% over the control reading by the fifth week, reaching the level of 124.4 ± 1.2 mm Hg. By the end of the seventh week following 60% reduction in renal mass, the systolic blood pressure reached a level of only 125.2 ± 1.3 mm Hg, an increase of only 8% (the usual increase in blood pressure observed in untouched rats due to the aging process). These findings show that a 60% reduction in

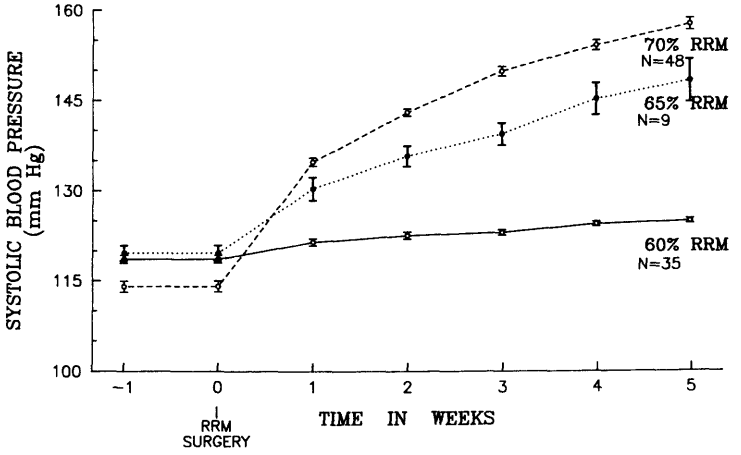

FIG. 1. Average weekly systolic blood pressure (tail plethysmography) before and after 60, 65, and 70% reduction in renal mass (RRM) in rats consuming salt-free diet (0.02% NaCl) and drinking 1% saline solution *ad libitum*. Diet and saline consumption followed RRM surgery at time 0.

renal mass places rats on the threshold for the development of hypertension.

Figures 2 and 3 show the effects on blood pressure of treatment with ANF antibody, control rabbit serum, and normal rabbit serum in normotensive rats with 60% reduction in renal mass. Blood pressure increased in rats receiving ANF antibody from 126.0 ± 1.0 to 158.4 ± 2.7 mm Hg by the 12th day (Fig. 2). Most of the increase occurred over the period 2–7 days (Figs. 2 and 3). Following the withdrawal of the ANF antibody treatment, the blood pressure returned to the control level in 5–6 days (Fig. 3). Injection of the normal rabbit serum and its withdrawal, as well as injection of serum from rabbits treated identically to those providing and without an X-80 anti-ANF antibody titer, had no effect on blood pressure (Figs. 2 and 3). The injection of anti-Ac-β-End or anti-CCK antibodies also had no effect on blood pressure in 60% reduced renal mass rats on a sodium-free diet drinking 1% saline.

The control (before the beginning of the treatment) body weights of ANF antibody and normal rabbit serum-treated rats in Fig. 2 (417.9 ± 8.9 and 400.0 ± 18.08 g, respectively) were not significantly different. However, at the end of the treatment period the ANF antibody-treated rats showed an increase in body weight from 417.9 ± 8.9 to 444.9 ± 8.9 g, $P < 0.05$, whereas the normal serum-treated rats did not show a significant increase in body weight (400.4 ± 18.08 to 413.8 g, $P > 0.05$). The serum constituents Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, osmolality, creatinine, BUN, and proteins were not different in these two groups of rats at the end of treatment with ANF antibody or normal rabbit serum.

Discussion. We (26, 30) and others (31, 32) have previously reported that reduction of renal mass by 70% or more in rats drinking 1% saline induces a low-renin, volume-dependent (26) type of hypertension. In contrast, rats are able to maintain normal blood pressure levels following a 60% reduction of renal mass (Fig. 1). However, any slight further reduction of renal mass (i.e., 5%) induces hypertension in these animals. These findings suggest that although 60% reduced renal mass rats are normotensive, their capacity to regulate blood pressure is strained nearly to the limit. The *in vivo* blockade of endogenous ANF by administration of ANF antibody induced hypertension in these animals (Figs. 2 and 3) and withdrawal of ANF antibody normalized the blood pressure in these animals (Fig. 3). The increase in blood pressure and its normalization following injection and withdrawal of ANF antibody, respectively, cannot be atributed to any nonspecific effects of the immunization procedure. Neither hyperimmune sera, developed

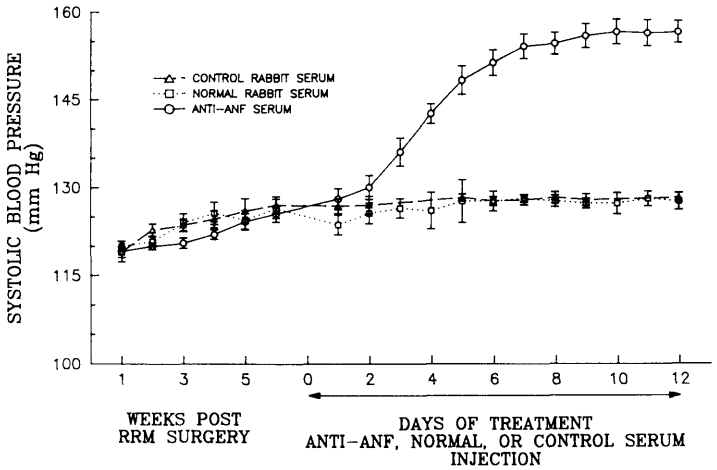

FIG. 2. Effect of ANF antibody (open circles, $N = 8$) normal rabbit serum (open squares, $N = 6$), or control rabbit serum (open triangles, $N = 10$) treatment on blood pressure in normotensive rats with 60% reduction in renal mass, consuming salt-free diet and drinking 1% saline *ad libitum*.

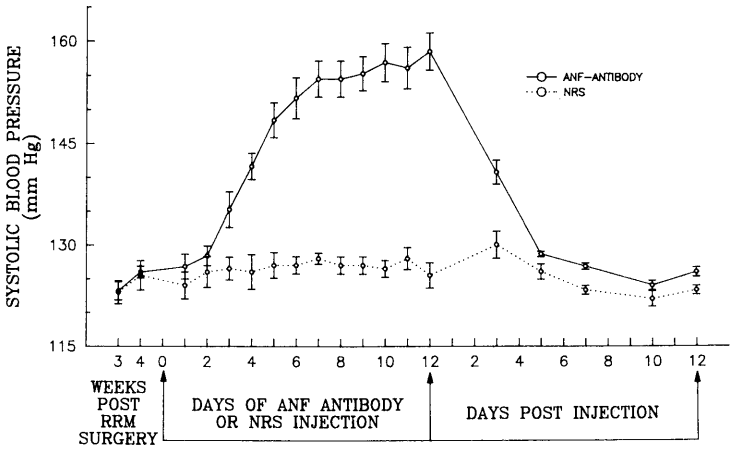

FIG. 3. Effect of ANF antibody or normal rabbit serum (NRS) treatment and its withdrawal on blood pressure in normotensive rats with 60% reduction in renal mass, consuming salt-free diet and drinking 1% saline *ad libitum* ($N = 5$).

against the thyroglobulin carrier, nor antisera developed against other peptides unrelated to ANF, had effects on blood pressure. Thus, the lowering of blood pressure by anti-ANF antisera appears to be specific to the immunoneutralization of endogenous atrial peptides. Although immunoglobulins and immune responses can alter, nonspecifically renal function and blood pressure, this was not a component underlying the observations reported here. The absence of a significant difference in serum creatinine, BUN, or protein in rats treated with anti-ANF sera or normal rabbit sera further supports this conclusion. These results indicate that rats with 60% reduction in renal mass and drinking 1% saline maintain normal blood pressure, in part, through the actions of endogenous ANF. The evidence indicating that ANF has potent natriuretic and diuretic actions (1–4) supports the conclusion that blockade of endogenous ANF is equivalent to further decrease in renal excretory function or further reduction in renal mass. This may explain a significant increase in body weight in rats treated with ANF antibody but not in rats treated with normal rabbit serum. The elevation of blood pressure following the treatment with the ANF antibody in 60% reduced renal mass rats may also have been due to blockade of a vasorelaxant effect of endogenous ANF. Several investigators have reported a vasorelaxant effect of atrial tissue extracted ANF or synthetic atriopeptins *in vitro*. The vasodepressor effect of intravenously administered atrial tissue extracted ANF or synthetic atriopeptin is pronounced only in hypertensive test animals (15, 16, 18). In the normotensive animals, however, the vasodepressor effect is either absent (4, 15–17) or very small in magnitude (1, 18, 19). Thus, it appears that homeostatic mechanisms normally involved counter the depressor actions of atrial peptides in normotensive animals in blood pressure regulation. This view is supported by the finding that the vasodepressor effect of ANF is enhanced following sinoatrial denervation (33, 34). Therefore, in rats with 60% reduction in renal mass, the ability of these homeostatic mechanisms to regulate blood pressure may be compromised to the point that the blood pressure effects of ANF antibody, and its withdrawal, are readily demonstrated.

In most studies in which a depressor response to ANF has been detected, in both hypertensive and normotensive state, it has been of short duration. Following a bolus injection of ANF, the fall in blood pressure, like the natriuresis and diuresis, is usually transient, lasting 20–30 min. In studies involving an ANF intravenous infusion, the fall in blood pressure usually lasts only for the duration of the infusion and rapidly re-

turns to normal upon termination of the infusion. In the present study, the treatment with ANF antibody produced a prolonged elevation of blood pressure and on withdrawal of ANF antibody, it took about 4 days for the blood pressure to return to normal. This suggests that endogenous ANF probably plays an important role in the regulation of blood pressure on a chronic basis in this setting.

1. DeBold AJ, Borenstein HB, Veress AT, Sonnenberg H. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. Life Sci **28**:89–94, 1981.
2. Garcia R, Cantin M, Thibault G, Ong H, Genest J. Relationship of specific granules to the natriuretic and diuretic activity of rat atria. Experientia **38**:1071–1073, 1982.
3. Tripodo NC, MacPhee AA, Cole Fe, Blakesley HL. Partial characterization of a natriuretic substance in rat atrial tissue. Proc Soc Exp Biol Med **170**:502–508, 1982.
4. Pamnani MB, Clough DL, Chen JS, Link WT, Haddy FJ. Effect of rat atrial extract on sodium transport and blood pressure in the rat. Proc Soc Exp Biol Med **176**:123–131, 1984.
5. Currie MG, Geller DM, Cole BR, Siegel NR, Fok KF, Adams SP, Eubanks SR, Galluppi GR, Needleman P. Purification and sequence analysis of bioactive atrial peptides (atriopeptins). Science **223**:67–69, 1984.
6. Kangawa K, Matuso H. Purification and complete amino acid sequence of α-human atrial natriuretic polypeptide (α-h ANP). Biochem Biophys Res Commun **118**;131–139, 1984.
7. Flynn TG, deBold ML, deBold AJ. The amino acid sequence of an atrial peptide with potent diuretic and natriuretic properties. Biochem Biophys Res Commun **117**:859–865, 1983.
8. Misono KS, Fukumi H, Grammer HT, Inagami T. Rat atrial natriuretic factor: Complete amino acid sequence and disulfide linkage essential for biological activity. Biochem Biophys Res Commun **119**:524–529, 1984.
9. Geller DM, Currie MG, Wakitani K, Cole BR, Adams SP, Fok KF, Siegel NR, Eubanks SR, Galluppi GR, Needleman P. Atriopeptins: A family of potent biologically active peptides derived from mammalian atria. Biochem Biophys Res Commun **120**:333–338, 1984.
10. Seidah NG, Lazuri C, Chretien M, Thibault G, Garcia R, Cantin M, Genest J, Nutt RF, Brady SF, Lyle TA, Paleveda WJ, Colton CD, Ciccerone TM, Veber DF. Amino acid sequence of homologous rat atrial peptides: Natriuretic activity of native and synthetic form. Proc Natl Acad Sci USA **81**:2640–2644, 1984.
11. Atlas SA, Kleinert HD, Camargo MJ, Januszewicz A, Sealey JE, Laragh JH, Schilling JW, Lewicki JA, Johnson LK, Maack T. Purification, sequencing and synthesis of natriuretic and vasoactive rat atrial peptide. Nature (London) **309**:717–719, 1984.
12. Wakitani K, Currie MG, Geller MG, Needleman P. Vasodilator properties of a family of bioactive atrial peptides in isolated perfused rat kidney. J Lab Clin Med **105**:349–352, 1985.
13. Currie MG, Geller DM, Cole BR, Boylan JG, Wu YS, Holmberg SW, Needleman P. Bioactive cardiac substances: Potent vasorelaxant activity in mammalian atria. Science **221**:71–73, 1983.
14. Kleinert HD, Maack T, Atlas SA, Januszewicz A, Sealey JE, Laragh JH. Atrial natriuretic factor inhibits angiotensin, norepinephrine, and potassium induced vascular contractility. Hypertension (Suppl 1):L-143–L-147, 1984.
15. Garcia R, Thibault G, Gutkowska J, Horky K, Hamet P, Cantin M, Genest J. Chronic infusion of low doses of atrial natriuretic factor (ANF Arg 101-Tyr 126) reduces blood pressure in conscious SHR without apparent changes in sodium excretion. Proc Soc Exp Biol Med **19**:396–401, 1985.
16. Debinski W, Kuchel O, Garcia R, Bun NT, Racz K, Cantin M, and Genest J. Atrial natriuretic factor inhibits the sympathetic nervous activity in one kidney, one clip hypertension in the rat. Proc Soc Exp Biol Med **181**:173–177, 1986.
17. Hintze TH, Currie MG, Needleman P. Atriopeptins: Renal specific vasodilators in conscious dogs. Amer J Physiol **248**:H587–H591, 1985.
18. Volpe M, Odell G, Kleinert HD, Muller F, Camargo MJ, Laragh JH, Maack T, Vaughan ED, Atlas SA. Effect of atrial natriuretic factor on blood pressure, renin and aldosterone in Goldblatt hypertension. Hypertension (Suppl I):I-43–I-48, 1985.
19. Breuhaus BA, Saneii HH, Brandt MA, Chimoskey JE. Atriopeptin II lowers cardiac output in conscious sheep. Amer J Physiol **249**:R776–R780, 1985.
20. Sonnenberg H, Milojevic S, Chong CK, Veress AT. Atrial natriuretic factor: Reduced cardiac content in spontaneously hypertensive rats. Hypertension **5**:672–675, 1983.
21. Hirata Y, Ganguli M, Tobian L, Iwai J. Dahl S rats have increased natriuretic factor in atria but are markedly hyporesponsive to it. Hypertension **6**(Suppl I):I-148–I-155, 1984.
22. Snajdar RM, Rapp JP. Atrial natriuretic factor in Dahl rats atrial content and renal and aortic responses. Hypertension **7**:755–782, 1985.
23. Haas M, Zamir N, Zukowska-Grojec Z. Plasma levels of atrial natriuretic polypeptide in plasma of hypertensive Dahl salt-sensitive rats. Biochem Biophys Res Comm **137**:876–883, 1986.

24. Imada T, Takayanagi R, Inagami T. Changes in the content of atrial natriuretic factor with the progression of hypertension in spontaneously hypertensive rats. Biochem Biophys Res Commun **133**:759–765, 1985.
25. Snajdar R, Rapp JP. Elevated atrial natriuretic polypeptide in plasma of hypertensive Dahl salt-sensitive rats. Biochem Biophys Res Commun **137**:876–883, 1986.
26. Huot SJ, Pamnani MB, Clough DL, Haddy FJ. The role of sodium intake, the Na^+-K^+ pump and a ouabain-like humoral agent in the genesis of reduced renal mass hypertension. Amer J Nephrol **3**:92–99, 1983.
27. Mueller GP, Hom GH, Haddy FJ, Pamnani MB. Regional distribution of immunoreactive atriopeptin III (iAPIII) in the rat. Fed Proc **44**:631, 1985.
28. Bonsnes RW, Tausky HH. On the colorimetric determination of creatinine by the Jaffe reaction. J Biol Chem **158**:581–591, 1945.
29. Davidson I, Wells BB. Clinical diagnosis by laboratory methods. Saunders, Philadelphia, 13th ed, 1962.
30. Pamnani MB, Huot SJ, Buggy J, Haddy FJ. Demonstration of a humoral inhibitor of the Na^+-K^+ pump in some models of experimental hypertension Hypertension **3**(Suppl. II):II-96–II-101, 1981.
31. Ylitalo P, Gross F. Hemodynamic changes during the development of sodium induced hypertension in subtotally nephrectomized rats. Acta Physiol Scand **106**:447–455, 1979.
32. Muirhead EE, Rightsel WA, Leach BE, Byers CW, Pitcock JA, Brooks B. Reversal of hypertension by transplants and lipid extracts of cultured renomedullary interstitial cells. Lab Invest **35**:162–172, 1977.
33. Thoren P, Morgan D, O'Neill TP, Needleman P, Mark A, Brody MJ. Atrial natriuretic factor activates vagal afferents in rats. Fed Proc **44**:1886, 1985.
34. Koyoma S, Nishida Y, Hosomi H, Abe Y. Participation of baroreceptor reflexes in blood pressure and sympathetic nerve responses to a synthetic human atrial natriuretic peptide in anesthetized dogs. Eur J Pharmacol **127**:43–48, 1986.

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