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Research Article

Atrial natriuretic peptide (ANP) specifically stimulates particulate guanylate cyclase, and cyclic guanosine monophosphate (cGMP) has been recognized as its second messenger. Spontaneously hypertensive rats (SHR) have elevated plasma ANP levels, but manifest an exaggerated natriuretic and diuretic response to exogenous ANP when compared to normotensive strains. In isolated glomeruli, the maximal cGMP response to ANP corresponds to a 12- to 14-fold increase over basal levels in normotensive strains (Wistar 13 +/- 2; Wistar-Kyoto 12 +/- 2; Sprague-Dawley 14 +/- 2) while a maximal 33 +/- 3-fold elevation occurs in SHR ($P < 0.001$). This hyperresponsiveness of cGMP is reproducible in intact glomeruli from SHR from various commercial sources. Furthermore, this abnormality develops early in life, even before hypertension is clearly established, and persists despite pharmacological modulation of blood pressure, indicating that it is a primary event in hypertension. In vitro studies have revealed a higher particulate guanylate cyclase activity in membranes from glomeruli and other tissues from SHR. This increase is not accounted for by different patterns of ANP binding to its receptor subtypes between normotensive and hypertensive strains, as assessed by competitive displacement with C-ANP102-121, an analog which selectively binds to one ANP receptor subtype. The hyperactivity of particulate guanylate cyclase in SHR and its behavior under basal, ligand (ANP), and detergent-enhanced conditions could be attributed either to increased [...]

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Increased Cyclic Guanosine Monophosphate Production and Overexpression of Atrial Natriuretic Peptide A-Receptor mRNA in Spontaneously Hypertensive Rats

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Abstract

Atrial natriuretic peptide (ANP) specifically stimulates particulate guanylate cyclase, and cyclic guanosine monophosphate (cGMP) has been recognized as its second messenger. Spontaneously hypertensive rats (SHR) have elevated plasma ANP levels, but manifest an exaggerated natriuretic and diuretic response to exogenous ANP when compared to normotensive strains. In isolated glomeruli, the maximal cGMP response to ANP corresponds to a 12- to 14-fold increase over basal levels in normotensive strains (Wistar 13±2; Wistar-Kyoto 12±2; Sprague-Dawley 14±2) while a maximal 33±3-fold elevation occurs in SHR ($P < 0.001$). This hyperresponsiveness of cGMP is reproducible in intact glomeruli from SHR from various commercial sources. Furthermore, this abnormality develops early in life, even before hypertension is clearly established, and persists despite pharmacological modulation of blood pressure, indicating that it is a primary event in hypertension.

In vitro studies have revealed a higher particulate guanylate cyclase activity in membranes from glomeruli and other tissues from SHR. This increase is not accounted for by different patterns of ANP binding to its receptor subtypes between normotensive and hypertensive strains, as assessed by competitive displacement with C-ANP¹⁰²⁻¹²¹, an analog which selectively binds to one ANP receptor subtype. The hyperactivity of particulate guanylate cyclase in SHR and its behavior under basal, ligand (ANP), and detergent-enhanced conditions could be attributed either to increased expression or augmented sensitivity of the enzyme. Radiation-inactivation analysis does not evoke a disturbance in the size of regulatory elements normally repressing enzymatic activity, while the expression of particulate guanylate cyclase gene using mutated standard of A- and B-receptors partial cDNAs, quantified by polymerase chain reaction (PCR) transcript titration assay, manifests a selective increase of one guanylate cyclase subtype. Our data suggest that in hypertension, genetic overexpression of the ANP A-receptor subtype is related to the exaggerated biological response to ANP in this disease. (*J. Clin. Invest.* 1993. 92:2499-2508.)
Key words: Glomeruli • atrial natriuretic peptide • guanylate

cyclase • spontaneously hypertensive rats • cyclic guanosine monophosphate • quantitative polymerase chain reaction

Introduction

Atrial natriuretic peptide (ANP),¹ a hormone synthesized in specific granules of the heart, elicits multiple physiological responses apparently mediated via cyclic GMP (cGMP; for review, see references 1-5). Some of its most striking biological effects are the natriuresis and diuresis it produces. Despite numerous investigations of its renal mechanisms of action, the exact processes involved are still not fully understood. High concentrations of particulate guanylate cyclase and cGMP levels elevated by ANP have been noted in glomeruli (6-9), which possess an abundance of specific ANP receptors (6, 10-14).

The existence of distinct ANP binding sites has been demonstrated (15-17). The larger (130-kD) ANP receptor type is a glycoprotein linked to particulate guanylate cyclase with rigorous binding specificity (18-21). Isolation, sequencing, cloning, and expression of the protein from various species have shown that guanylate cyclase is an integral part of the molecule (22-25). Two subtypes of guanylate cyclase-related receptors (ANP-A and ANP-B receptors) have been cloned with different ligand binding affinities despite overall structural homology (24, 26, 27). Elucidation of the gene structure of the ANP A-receptor, responding preferentially to ANP, has been achieved (28). Both receptor subtypes have a large extracellular NH₂-terminal domain, a putative single membrane spanning sequence and an intracellular region containing both a putative kinase-like regulatory domain required for signalling via ANP (29), and a guanylate cyclase catalytic domain (24, 25) responsible for synthesis of cGMP, the effector of ANP. Another receptor type, known as the C-receptor, has a molecular mass of 120 kD on SDS-PAGE and can be reduced to 65 kD with β -mercaptoethanol (30). It has a high affinity for the ligand and is widely distributed (30). This truncated homodimeric glycoprotein has only 37 amino acids for its intracellular domain (30) and is therefore not linked to catalytic guanylate cyclase activity (20, 30). Its role as a clearance or buffer receptor (31) is closely related to internalization of the receptor-ligand complex, recycling and hydrolyzing ANP (32). However, the down-regulation of ANP receptors may require prior ANP-guanylate cyclase receptor occupancy (33).

ANP has been postulated to play a role in the development of arterial hypertension and in the counterregulation of increased blood pressure. There are many reports of elevated plasma ANP levels in human essential hypertension (34-38), even in correlation with the extent of left ventricular and auri-

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1. Abbreviations used in this paper: ANP, atrial natriuretic peptide; SHR, spontaneously hypertensive rats.

cular abnormalities (39), and also in healthy young people with a family history of hypertension as well as in SHR (40–44). Plasma ANP levels have been reported to be increased in different forms of experimental hypertension (45–48). However, some authors have not found elevated ANP levels in patients with mild, age-adjusted hypertension (49–51) or in young SHR (52).

Down-regulation of ANP receptors has been reported in vitro in vessels as well as in cultured smooth muscle cells, contrasting with normal or even increased cGMP production (53–57). No difference in receptor density or affinity is observed in glomeruli of young SHR (58, 59), while older animals with heightened blood pressure reportedly demonstrate a reduced receptor population (59, 60). Changes occur in other organ systems as well (44, 61, 62). Furthermore, the number of binding sites is modified by alterations in blood pressure. Data on binding affinity in these studies are conflicting. In other models of experimental hypertension, binding affinity is increased in the early hypertensive phase (63), while receptor density is unchanged or diminished in later periods (45, 47, 48, 64).

Since hypertensive animals appear to have normal to elevated endogenous plasma ANP levels with decreased binding capacity, it is surprising that ANP infusions have an increased or prolonged effect on diuresis, natriuresis, and blood pressure in hypertensive rats and monkeys (65–69) as well as in humans with essential hypertension (70–72). We therefore evaluated the ANP response of guanylate cyclase in normotensive rats and SHR in studies aimed at elucidating its mechanism in this model of hypertension.

Methods

Materials. Male rats obtained from Taconic Farms Inc., Germantown, NY (WKY, SHR), Charles River, St-Constant, Que., Canada, and Harlan Sprague Dawley Inc., Indianapolis, IN, were kept at room temperature under a 12 h:12 h dark/light cycle. They were fed normal Purina rat chow and allowed free access to tap water. Synthetic rat ANP^{99–126} and leupeptin were purchased from Institut Armand-Frapier, Laval, Que., Canada. C-ANP^{102–121}-des[Gln¹¹⁶, Ser¹¹⁷, Gly¹¹⁸, Leu¹¹⁹, Gly¹²⁰]ANP-NH₂ (C-ANP) was generously donated by Bio-Mega, Chomedey, Laval, Que., Canada. Carrier-free ¹²⁵I-Na was acquired from Amersham Corp., Oakville, Ont., Canada; disuccinimidyl suberate (DSS) and Triton X-100 were procured from Pierce, Rockford, IL, USA. Electrophoresis reagents and molecular weight standards were obtained from Bio-Rad, Mississauga, Ont., Canada. Lactoperoxidase and bovine gamma-globulin came from Sigma, St. Louis, MO.

Tissue preparations. Male rats were decapitated between 8 to 10 a.m. to exclude a possible effect of diurnal variations. Their kidneys were rapidly removed and placed in gas-modified (95% O₂/5% CO₂) Krebs solution, pH 7.4, at 4°C. The cortex was separated, cut into slices, and gently ground successively through 250- and 150- μ m molecular sieves. The pass-through fraction was washed three times with Krebs solution on a 70- μ m mesh. The supernatant was then centrifuged three times at 400 g for 3 min. The purity of the suspensions, verified by phase contrast microscopy, was over 95%. Protein concentrations of glomerular preparations were measured by the method described by Lowry et al. (73).

Whole lungs and livers were also removed, while the renal papilla and zona glomerulosa were dissected from the kidney and adrenal cortex respectively. The tissues were homogenized with a Potter-Elvehjem homogenizer for large preparations or with a Dounce homogenizer for smaller samples in 5 vols of ice-cold 50 mM Hepes buffer, pH 7.4, containing 1 mM EDTA, 10 μ g/ml leupeptin, and 0.1 mM PMSF. After centrifugation at 30,000 g for 30 min, the pellets were resus-

ended and centrifuged a second time. Washed pellets were resuspended in the same buffer at a protein concentration of \sim 20 mg/ml.

Stimulation of cGMP production in glomeruli. Isolated glomeruli (6) in the amount of 400–500 μ g were incubated in 400 μ l Krebs solution for 15 min at 37°C and for 2 additional min with 500 μ M of the phosphodiesterase inhibitor, methylisobutylxanthine (MIX; reference 9). Acetic acid (0.01 N) or increasing concentrations of ANP (10⁻¹⁰ to 10⁻⁶ M) were then added. The reaction was stopped after 90 s with 1N perchloric acid. The samples were rapidly frozen in liquid nitrogen and kept at -35°C until sonication, extraction, and cGMP measurement by RIA, as described previously (74, 75).

Solubilization of membranes. Glomeruli and papilla were isolated as described above. For binding and crosslinking studies, the membrane fractions were solubilized with 3% Triton X-100 for 60 min at 4°C in 50 mM Hepes buffer, pH 7.4, containing 1 mM EDTA, 50 μ g/ml leupeptin, 0.1 mM PMSF, and 0.6 M KCl. The samples were then centrifuged at 105,000 g for 30 min and resuspended in the same buffer. Protein content was measured by Peterson's method (76).

Receptor binding assay. ANP^{99–126} was iodinated with 1 mCi of ¹²⁵I-labeled Na by the lactoperoxidase method and purified by HPLC on a C₁₈ μ -Bondapak column, as described previously (77). For ANP binding assay, glomeruli were isolated and membranes solubilized as detailed above. Briefly, 50 μ g of protein were incubated with ¹²⁵I-labeled ANP (10,000 cpm) for 60 min at 22°C in 100 mM phosphate buffer, pH 7.4, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 5 mM MgCl₂, 0.5 mM PMSF, 0.4% BSA and 0.1% Triton X-100 in the absence or presence of increasing concentrations of cold ANP^{99–126} or C-ANP^{102–121} in a final volume of 100 μ l. Nonspecific binding was determined in the presence of 1 μ M of unlabeled ANP^{99–126}. At the end of incubation, 25 μ l of bovine gamma-globulin (0.3%) and 1 ml of polyethyleneglycol (PEG; 16%) in phosphate buffer, pH 7.4, were added. The incubation mixture was immediately poured onto polyethylenimine-treated (0.3%, 30 min) Whatman GF/C fiberglass filters, rinsed three times with 1 ml PEG, and its radioactivity was counted with a Gamma counter.

Affinity cross-linking with ¹²⁵I-ANP. Triton X-100-solubilized glomerular or papillar proteins (500 μ g) were incubated with 0.1 nM ¹²⁵I-labeled ANP (5 \times 10⁶ cpm) for 60 min at 22°C in 1 ml of sodium phosphate buffer, pH 7.4, containing 0.1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 5 mM MgCl₂ in the absence or presence of 0.1 μ M unlabeled C-ANP^{102–121} or ANP^{99–126}. After incubation, the protein-bound radioactivity was isolated by gel filtration on Sephadex G-50 (0.5 \times 14 cm), and the cross-linking reagent DSS was added at a final concentration of 0.5 mM and incubated for 15 min at 22°C. The final reaction was quenched by 50 mM ammonium acetate. Bound ¹²⁵I-labeled ANP was detected by autoradiography after SDS-PAGE.

Guanylate cyclase assay. Particulate guanylate cyclase activity was measured in membranes of various tissues, as described previously (78). The reaction mixture (0.1 ml) contained 9.25 mg/ml phosphocreatine, 2.5 mg/ml creatine phosphokinase, 2 mM MIX, 10 mM theophylline, 3 mM excess MnCl₂, 1 mM GTP-MnCl₂, and 50 mM triethanolamine buffer, pH 7.4, in the absence or presence of 1 μ M ANP. Membrane proteins or solubilized preparations were added, incubated at 37°C for 30 min, and the reaction was stopped with 2 ml of 30 mM hot EDTA. cGMP formed was measured by radioimmunoassay (74, 75).

Radiation-inactivation. Pulmonary membrane preparations were brought to 2 mg/ml of proteins and pre-incubated at 4°C for 10 min with 0.01 N acetic acid or with 0.5 μ M ANP, a dose which was shown in pilot experiments to fully activate guanylate cyclase. Control and ANP-treated samples were quickly aliquoted into Eppendorf tubes (200 μ l) and rapidly frozen in liquid nitrogen. Samples kept at -78°C with dry ice at exposure times varying between 0 and 24 h were irradiated in a Gammacell 220 irradiator (Atomic Energy of Canada Ltd., Ottawa, Ont., Canada) delivering a steady dose of about 1.35 Mrads/h (79–81). Residual guanylate cyclase activity assessed on rapidly-thawed samples was plotted semilogarithmically as a function of radiation dose. A line passing near 2 (100% activity) at 0 radiation dose was

constructed by least square analysis. The radiation-inactivation size (or RIS) of the functional unit studied was calculated by the equation

$$\log \text{RIS} = 5.89 - \log D_{37} - 0.0028t$$

where D_{37} is the radiation dose needed to decrease the function of a protein to 37% of its initial value (82) and t is the temperature during radiation (83).

Quantitative measurement of ANP A- and B-receptor mRNA transcripts, with regard to (a) preparation of mutant ANP A- and B-receptor cDNA and cRNA. To clone a fragment of ANP A-receptor cDNA, total RNA from rat pituitaries was extracted and transcribed into cDNA with M-MLV reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) primed with random hexamers according to the manufacturer's protocol. A 709 bp fragment was generated by 30 cycles of polymerase chain reaction (PCR) in a thermocycler (Perkin Elmer Cetus, Norwalk, CT) as described below. The sense and antisense primers were respectively: GGAGAGAACACAAGCTTATCTGG-AGG and AAAGTCGACCCCGGGAGGTCAGCCTCGAGTGGC-TACA. The sense primer from base 2712 (84) contained a HindIII site (underlined) while the antisense primer from base 3404 was extended in 5' with two sites, Sal I and Sma I. Using these sites, the PCR fragment was cloned into the plasmid pSP72.

A mutant ANP A-receptor cDNA fragment was obtained by PCR with oligos containing mismatched bases (G → T at 3027, G → C at 3028), creating an EcoRI site at position 3023 of ANP A-receptor cDNA. This mutated partial cDNA was cloned into pSP73. The mutant ANP B-receptor (795 bp) cDNA fragment, from bases 2425 to 3213 of the reported sequence (26) was obtained using the same strategy and the following oligos sense primer from 2489 GGT-GGTACCAGCATATTGGAC containing a KpnI site (underlined) and antisense from 3193 AAAAGATCTTACAGGAGTCCAGGAGGTCCT extended with a BglII site. Two point mutations (A → T at 2837 and G → C at 2839) were introduced by PCR primed with mismatched oligos to create an EcoRI site at position 2835. The mutant ANP-B cDNA fragment was then inserted into pSP72. Mutant cRNA molecules were then generated by *in vitro* transcription using the Ribo-probe II core system (Promega, Madison, WI). These mutant RNAs were employed as internal standards in reverse transcription and quantitative PCR or as exogenous RNAs in modified PCR aided transcript titration assays.² The cRNAs were stored in 1 μg/10 μl aliquots at -70°C.

(b) Reverse transcription and quantitative PCR for ANP A- and B-receptors mRNA. Total cellular RNA was processed from rat lungs by the guanidinium isothiocyanate procedure (85). Serial dilutions of mutant cRNA for the ANP A- or B-receptor were added to a fixed amount of tissue RNA, either before RNA extraction to determine the efficiency of extraction or before reverse transcription and PCR for the construction of titration curves. Fractions of 1 or 2 μg total RNA were dissolved in water containing 100 pmol random hexamers. After 10 min of denaturation at 70°C, the solution was put on ice and mixed with 200 U of superscript RNase H⁻ reverse transcriptase (Canadian Life Technologies, Burlington, Ont., Canada) in RT buffer (final concentration 50 mM Tris HCl, pH 8.3, at 23°C) containing 75 mM KCl, 10 mM DTT, 3 mM MgCl₂, and 0.5 mM dNTP for a total volume of 20 μl. After 1 h of incubation at 37°C, one half of the reaction products was used for PCR amplification with the same set of primers described above for A or B receptor separately. A reaction volume of 50 μl containing 50 mM Tris-HCl buffer, pH 8.4, 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM dNTP, 50 pmol of each primer, 5 U TAQ polymerase (Perkin Elmer Cetus), and 20 μCi ³²P-dCTP was incubated for 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 65°C, and 1 min 30 s of extension at 72°C. After PCR, 10 μl of each sample were digested at

37°C for 1 h with 10 U of EcoRI. The two sets of primers did not crossreact with the other subtype of ANP receptor cDNA (not shown). Digested and undigested samples were electrophoresed on 1.5% agarose gel and radioactive bands were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Statistics. The results are expressed as means±SEM. Comparisons were made by one- or two-way ANOVA with a posteriori comparisons according to Bonferroni's method, or by the unpaired Student's *t* test. Differences were considered to be significant at $P < 0.05$.

Results

Stimulation of cGMP production by ANP in glomeruli of normotensive and hypertensive rats. cGMP production was measured in glomeruli after 90 s incubation with ANP⁹⁹⁻¹²⁶ in three normotensive strains (WKY, Wistar, and Sprague-Dawley rats) and in SHR (Fig. 1). Previous experiments had shown that cGMP increased maximally after a 90-s incubation period (6). Basal cGMP levels were similar in all four strains. ANP concentrations lower than 10⁻¹⁰ M did not enhance cGMP generation over basal levels in glomeruli in any of these animals. At an ANP concentration of 10⁻⁹ M, cGMP levels in normotensive strains rose between 50 and 80% while there was a 120% elevation in hypertensive animals. At higher ANP concentrations, cGMP levels increased more than 33-fold in SHR, while in the normotensive strains, they did not exceed a 15-fold augmentation (Wistar 13±2; Wistar-Kyoto 12±2; Sprague-Dawley 14±2). ANOVA established that the difference between the normotensive and hypertensive strains was highly significant ($P < 0.001$) whereas cGMP elevations in glomeruli

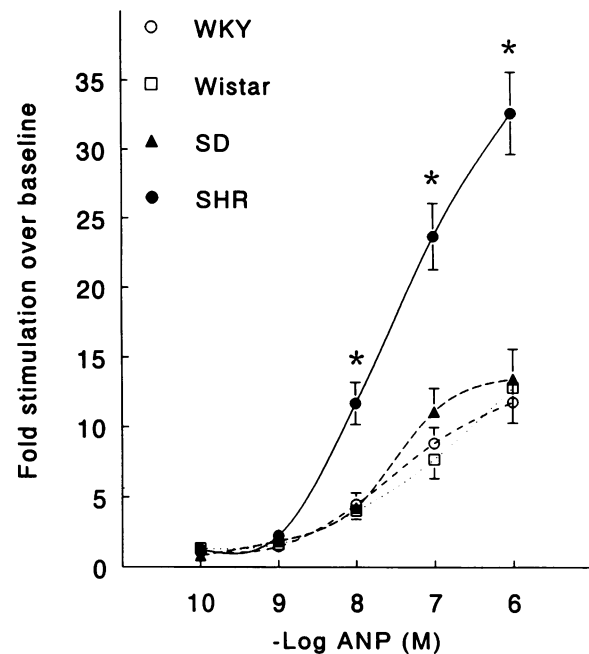


Figure 1. Effect of ANP on cGMP levels in glomeruli of SHR and different normotensive strains. Isolated glomeruli were incubated at 37°C with increasing concentrations of ANP in the presence of 0.5 mM MIX for 90 s. cGMP levels are expressed as means±SEM of fold increase ($n = 7$ animals of each strain). Baseline values of cGMP (pmol/mg protein) were similar in all strains: in WKY 1.74±0.21; in Wistar 1.83±0.19; in Sprague-Dawley 1.61±0.36; and in SHR 1.57±0.29. ANOVA demonstrated a significant difference in SHR cGMP response vs. all other strains ($P < 0.001$).

2. Fujio N., F. Bayard, F. Gossard, P. Hamet, and J. Tremblay. 1993. Differential messenger RNA expression of particulate guanylate cyclase A and B in rat tissues: Quantitative polymerase chain reaction analysis. (Manuscript submitted for publication.)

of the three normotensive strains did not differ significantly between each other.

Genetic heterogeneity has been demonstrated between animals from different commercial sources (86). We therefore evaluated the effect of a maximal dose of ANP ($1 \mu\text{M}$) on cGMP levels in isolated glomeruli from SHR and WKY originating from three major commercial sources in North America. Our results showed that the phenotype of the glomerular cGMP response to ANP was well conserved and that a 2- to 3-fold difference was observed in the glomerular cGMP increase between WKY and SHR from the three commercial sources (Table I). The difference between WKY and SHR preceded the phase of established hypertension. Thus, the fold increase of glomerular cGMP levels by ANP was 8 and 33 for WKY and SHR, respectively, at 6 wk of age, 11 and 40 at 8 wk, 13 and 31 at 10 wk, and 15 and 30 in 14-wk-old rats. However, the difference appeared to be less striking at an older age, a finding that can be partly explained by an enhanced response (nearly doubling) in young vs. adult WKY animals. This difference between WKY and SHR persisted despite modulation of blood pressure. Indeed, prevention of the blood pressure increase in SHR by combined hydralazine (10 mg/kg per d) and hydrochlorothiazide (200 mg/kg per d) therapy between the ages of 4 and 12 wk did not attenuate the exaggerated pattern of cGMP production, as compared to the stimulation observed in the normotensive control strain. In WKY, maximal stimulation was 16- and 20-fold over baseline in treated and untreated rats whereas in SHR, it was 46- and 41-fold over basal values under the same conditions (Fig. 2), despite 40 mmHg lower blood pressure ($132 \pm 2 \text{ mmHg}$ vs. $91 \pm 5 \text{ mmHg}$, respectively) in untreated and treated SHR.

Competitive ANP binding in glomeruli. The ANP binding characteristics of glomerular receptors in SHR, WKY, and Sprague-Dawley rats were determined by competitive binding of ^{125}I -labeled ANP with increasing doses of rat ANP (Fig. 3). The dissociation constants of the various strains were not significantly different (K_d in 10^{-10} M : WKY 3.0 ± 0.7 , Sprague-Dawley 4.6 ± 1.8 , SHR 4.1 ± 1.2). Receptor density (B_{max} in fmol/mg protein) was 520 ± 120 in WKY, 420 ± 120 in Sprague-Dawley, and 810 ± 230 in SHR (difference not significant).

Comparison of ANP⁹⁹⁻¹²⁶ and C-ANP¹⁰²⁻¹²¹ binding in glomeruli. C-ANP¹⁰²⁻¹²¹ is a truncated ANP analog which is more specific for the C-receptor. It induces glomerular cGMP production only at micromolar concentrations, with a maximal 80% increase in cGMP levels as compared to more than 10-fold for ANP⁹⁹⁻¹²⁶ in the same dose range (data not shown). Competitive inhibition of ^{125}I -ANP binding by C-ANP¹⁰²⁻¹²¹ and

Table I. Stimulation of cGMP Production by ANP ($1 \mu\text{M}$) in Glomeruli Isolated from Rats Originating from Different Commercial Sources

Strain	Commercial source		
	Charles River	Taconic	Harlan
WKY	10 ± 4	12 ± 5	14 ± 6
SHR	23	$29 \pm 10^*$	$40 \pm 1^*$

Glomeruli were isolated as described in Methods. Values represent the means \pm SEM of fold increase in cGMP levels over baseline. The number of experiments varied from 2 to 8. * $P < 0.05$.

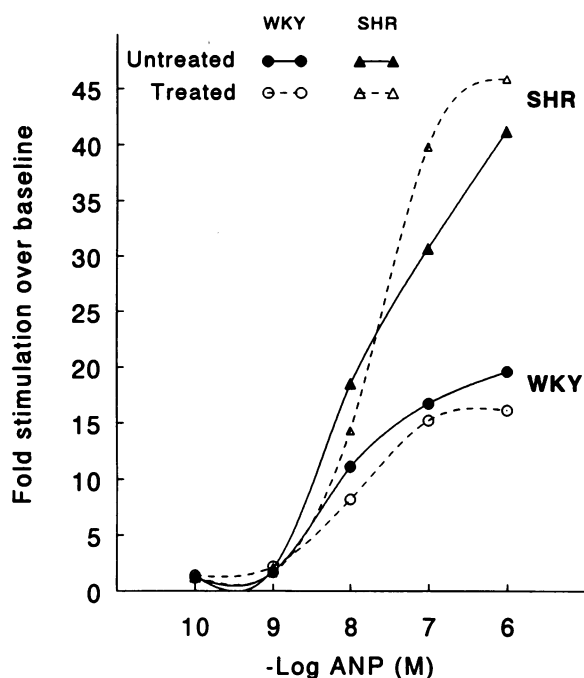


Figure 2. Effect of ANP on cGMP levels in WKY and SHR not treated or treated for 8 wk with hydralazine and hydrochlorothiazide to normalize blood pressure. The results are expressed as fold increase over basal levels.

ANP⁹⁹⁻¹²⁶ can be used as an indicator of the proportion of guanylate cyclase-related (ANP A- plus B-) receptors. In glomeruli from WKY, C-ANP displaced a maximum of 84% of ^{125}I -labeled ANP, indicating that the remaining 16% was not

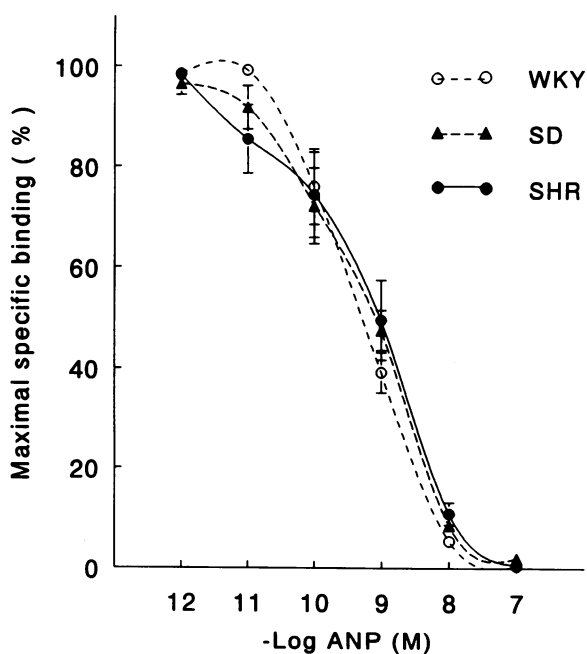


Figure 3. Competition for ^{125}I -labeled ANP binding to isolated glomeruli from SHR, WKY, and Sprague-Dawley rats. Values are shown as means \pm SEM ($n = 7$ animals of each strain) in % of maximal specific binding. Nonspecific binding was obtained by incubation with $1 \mu\text{M}$ rat ANP.

bound to the ANP C-receptor (Fig. 4). When the same type of experiment was performed in glomeruli from SHR, maximal displacement was 82% at 10^{-7} M C-ANP, suggesting that 18% of the ANP receptors were not ANP C-receptors. These results indicate that in glomeruli, about 80% of ANP receptors are of the C-receptor type and that guanylate cyclase vs. C-receptor distribution is similar between WKY and SHR glomeruli.

Affinity cross-linking of 125 I-labeled ANP in glomeruli and papillae from SHR and WKY rats. As described earlier (15, 18), affinity cross-linking in various tissues with 125 I-labeled ANP specifically labels 65- and 130-kD proteins. The high molecular weight form corresponds to the receptor containing particulate guanylate cyclase (15, 18). The 65-kD protein is the ANP C-receptor that does not contain any intracellular catalytic activity. In glomeruli isolated from either WKY or SHR, both 130- and 65-kD proteins were labeled (Fig. 5 A, lanes 1 and 4). Only the 65-kD protein was competed for by C-ANP (10^{-7} M; Fig. 5 A, lanes 2 and 5) while ANP⁹⁹⁻¹²⁶ displaced both bands (Fig. 5 A, lanes 3 and 6) in both strains. In both SHR and WKY papillae, affinity cross-linking with 125 I-labeled ANP labeled only the 130-kD protein (Fig. 5 B, lanes 1 and 4)

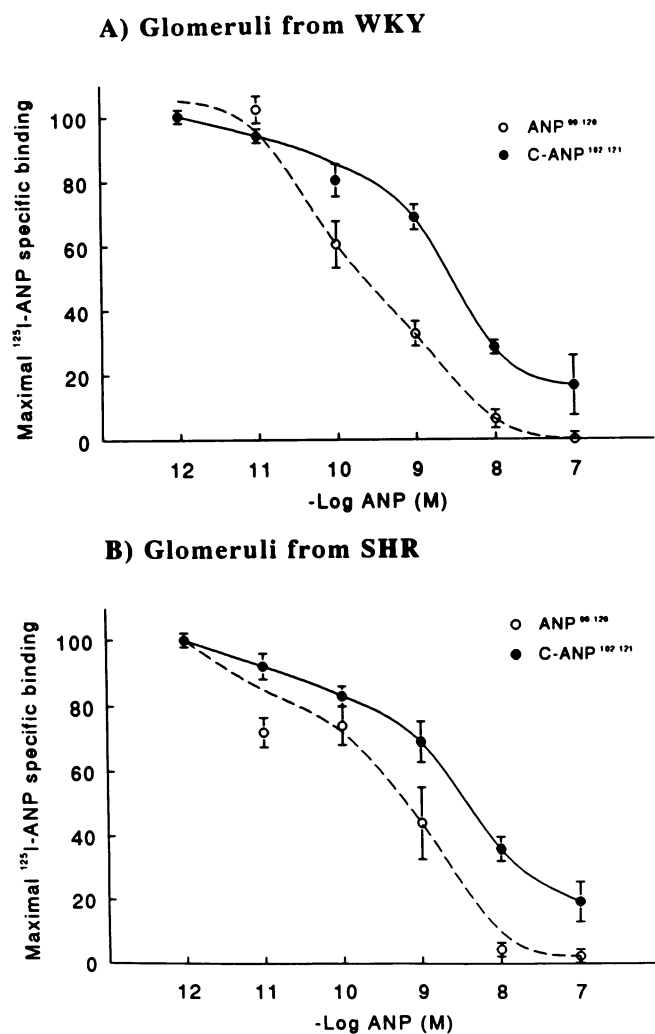


Figure 4. Competition of rat ANP⁹⁹⁻¹²⁶ and C-ANP¹⁰²⁻¹²¹ for 125 I-labeled ANP binding to isolated glomeruli from WKY and SHR. Values are expressed as means \pm SEM of maximal specific binding. Non-specific binding was obtained by incubation with 1 μ M rat ANP.

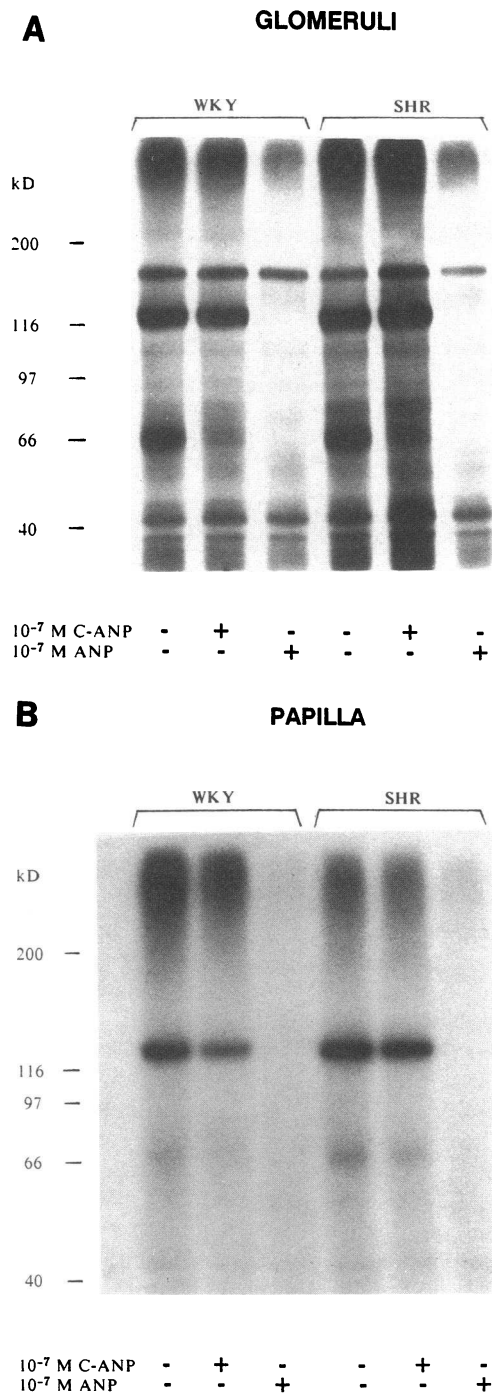


Figure 5. Affinity cross-linking with 125 I-labeled ANP. Triton X-100-solubilized glomerular (A) or papillar membranes (B) from WKY and SHR were incubated with 125 I-labeled ANP in the absence (lanes 1 and 4) or presence (lanes 2 and 5) of 10^{-7} M unlabeled C-ANP¹⁰²⁻¹²¹ or 10^{-7} M unlabeled ANP⁹⁹⁻¹²⁶ (lanes 3 and 6), cross-linked with 0.5 mM DSS, and subjected to PAGE and autoradiography.

which was also displaced by ANP⁹⁹⁻¹²⁶ (10^6 M; Fig. 5 B, lanes 3 and 6) but not by the same dose of C-ANP (Fig. 5 B, lanes 2 and 5). In Fig. 5 A, the 175-kD band was considered to be nonspecific, since it was neither displaced by ANP⁹⁹⁻¹²⁶ nor by C-ANP. Although some of the experiments (as shown in Fig. 5 A and B) disclosed higher cross-linking of the radiolabeled li-

Table II. Guanylate Cyclase Activity in Various Organs of Normotensive (WKY) and Spontaneously Hypertensive Rats (SHR)

	Basal		ANP		Triton X-100	
	WKY	SHR	WKY	SHR	WKY	SHR
Lung	24±4	64±6*	52±11	116±12*	162±41	288±41*
Kidney						
–Glomeruli	19±3	51±22*	60±21	110±64*	359±102	617±180*
–Papilla	35±7	71±20*	104±15	141±29	408±219	329±114
Adrenal cortex	48±10	69±6*	106±18	118±20	475±87	432±83
Liver	8±2	8±1	11±3	10±1	33±4	38±1

Values expressed in pmol cGMP/min per mg prot. Data represent the means±SEM of determinations performed at three membrane protein concentrations from three different pooled samples (3 animals each, i.e., 9 animals of each strain). Membranes were incubated for 30 min in the absence (basal) or presence of either ANP (10^{-6} M) or Triton X-100 (3% vol/vol) (* $P < 0.05$ between two strains).

gand to the 130-kD protein band in SHR, as compared to WKY, the difference did not reach statistical significance ($n = 6$), pointing toward similar binding capacities in the two organs studied.

Guanylate cyclase activity. Guanylate cyclase activity was assessed directly in vitro in washed membranes prepared from various WKY and SHR organs (Table II). Higher specific activity of particulate guanylate cyclase was found in membranes from the kidney papilla, adrenal cortex, and lungs, but not in the liver of SHR compared to WKY controls. When present, the greatest difference between WKY and SHR was observed for basal guanylate cyclase activity within its membrane environment. The difference between WKY and SHR decreased from basal to ANP-stimulated and Triton X-100-stimulated conditions, being least evident under the two latter conditions in the papilla and adrenal cortex. These results suggest that the basal specific activity of particulate guanylate cyclase within membranes of most organ systems is higher in SHR.

Radiation-inactivation. We attempted to characterize the behavior of the protein within its membrane environment in lung preparations from SHR and WKY by radiation-inactivation. This tissue source was chosen for its availability and similarity of guanylate cyclase behavior to glomerular tissues under basal, ANP-, and detergent-enhanced conditions (Table II). Under basal conditions, there is an activation hump in lung preparations from WKY and SHR rather than immediate destruction of enzymatic activity at doses up to 10 Mrads, followed by linear exponential decay. As reported previously (87, 88), this pattern is best explained by the presence of an intramolecular inhibitory component repressing guanylate cyclase activity in the basal state. ANP stimulation significantly enhanced guanylate cyclase decay by increasing doses of radiation in a monoexponential manner, most likely by liberating or deactivating the regulatory element (Fig. 6). Using the formula described in Methods, we calculated a RIS of 46 kD in both basal and ANP-stimulated states. The values did not differ significantly between WKY and SHR. These results indicate that if the guanylate cyclase domain in the protein within its natural environment, or following stimulation by the ligand, is any different in SHR, it is below the determination capability of this method.

Quantitative assay of ANP receptor subtype mRNAs by PCR-aided transcript titration. Total RNA from lung was used for the quantitation of ANP receptor mRNA. It can be seen

from Table II that guanylate cyclase activity in the lung was well correlated with renal glomerular activity in both WKY and SHR under basal and ANP-induced conditions. Because the lung can be easily and rapidly dissected from the animal,

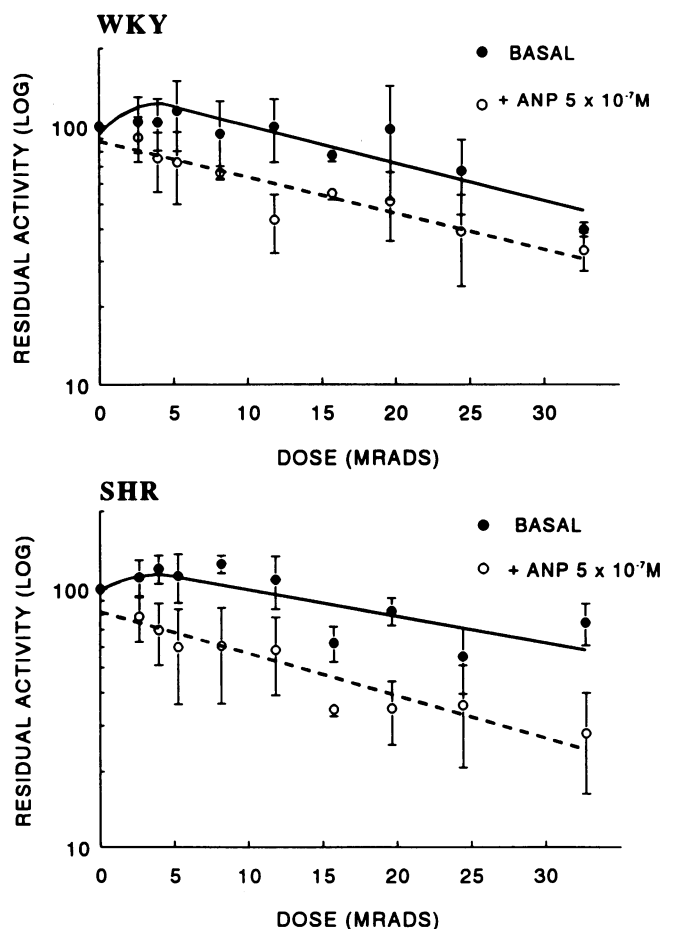


Figure 6. Radiation-inactivation of guanylate cyclase activity in lung membranes from WKY and SHR under basal conditions and ANP stimulation ($0.5 \mu\text{M}$). Data points are means±SEM of four separate experiments, each performed in triplicate. They are plotted as log % guanylate cyclase activity from baseline, representing nonirradiated samples (log 100% = 2) as a function of increasing irradiation doses. Full symbols, controls; empty symbols, ANP-treated (5×10^{-7} M).

and because mRNA for both receptors are more abundant in this organ (26), it served as a source of RNA. Fig. 7 A shows a representative PhosphorImager picture of PCR products from total lung RNA of WKY and SHR rats titrated with 2×10^5 , 10^6 , 5×10^6 , 2×10^7 and 10^8 molecules of mutated cRNAs of ANP-A and ANP-B receptors. After digestion with EcoRI, DNA fragments of 315 and 394 bp (lower bands) for ANP A-receptor and of 378 and 387 bp for ANP B-receptor were generated by mutant cDNAs. Titration curves were constructed for ANP A- and ANP B-receptor mRNAs, using a fixed amount of lung total RNA mixed with increasing amounts of mutated cRNA (0.20 to 100×10^6 molecules) before reverse transcription and PCR (Fig. 7 B). Percentages of ^{32}P radioactivity following EcoRI digestion in the upper band vs. the sum of the upper and lower bands were calculated; a 50% value corresponded to the absolute amount of ANP A- or B-receptor mRNA molecules per μg of total RNA. It was of relevance to analyze the ratio of A and B receptor mRNAs within each strain as well as the relative expression of A and B receptor mRNAs in WKY and SHR, respectively. Thus, the ANP B-receptor mRNA level was about threefold higher than that of ANP A-receptor in WKY rats, while in SHR both receptors were equally represented (Fig. 7 C). Furthermore, additional measurements in six different tissue preparations showed that ANP A-receptor mRNA levels were significantly higher in SHR than in WKY ($P < 0.01$), while the amount of mRNA of the ANP B-receptor was not significantly different between the two strains. In addition to Charles River SHR, these experiments were performed on rat strains from Taconic Farms with the same results.

Discussion

We report here a highly significant increase of cGMP production (about threefold) in response to ANP in glomeruli from SHR when compared to three normotensive rat strains. This phenotype is constant between SHR and WKY strains from three different suppliers and is present early in the life of SHR. Since blood pressure normalization does not affect this phenomenon, it has to be examined for its primary involvement in hypertension. As a first step, the present studies aimed at determining the mechanism of this increased cGMP production.

Data on ANP binding affinities and receptor densities are conflicting. Several authors have reported normal or diminished receptor density in glomeruli from SHR or in experimental hypertension (45, 48, 58–60). Our competitive binding studies did not show any significant difference in either total binding capacity or binding affinity between hypertensive and normotensive rats. This apparent contradiction may be due to the age of the animal studied and decrease in receptor density could be secondary to high blood pressure, since it is observed in experimental hypertension and older SHR. Furthermore, binding studies only demonstrate the presence of one binding site in spite of the existence of at least three different subtypes of ANP receptors (24, 26, 30). In glomeruli, we performed competitive displacement of ^{125}I -labeled ANP with C-ANP, an agonist specific for the C-receptor (31). C-ANP displaced 82 and 84% of specifically bound labeled ANP in WKY and SHR, respectively. This suggests that the remaining 16% and 18% of ^{125}I -labeled ANP not bound to the C-receptor were bound to ANP A- and B-receptors and that the proportion of guanylate

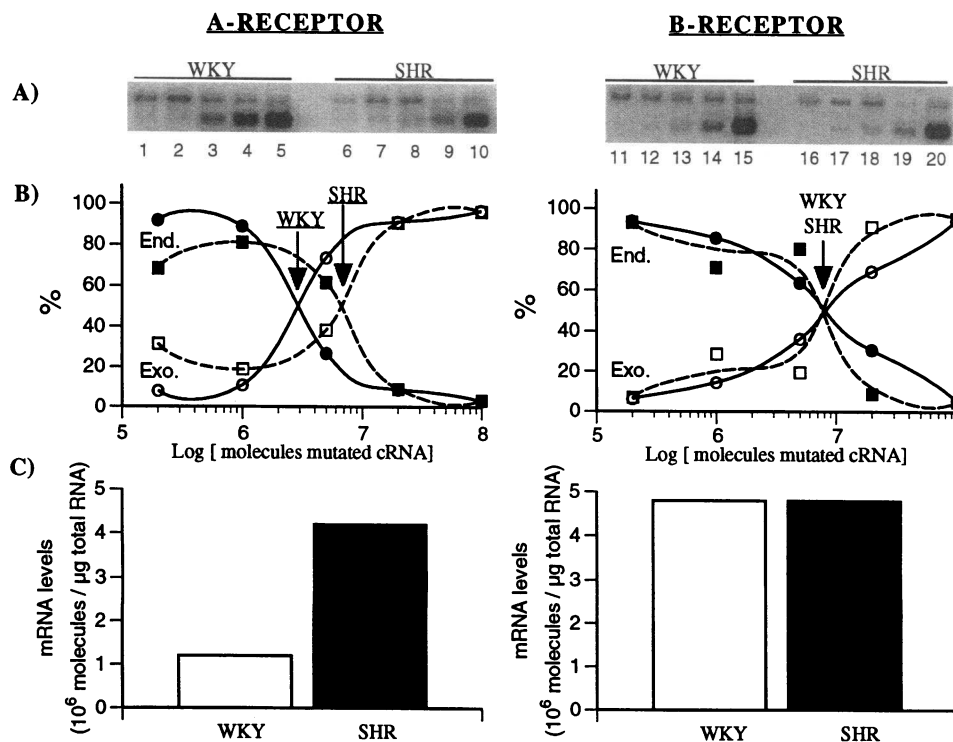


Figure 7. (A) Typical PCR transcript titration assay of ANP A- and ANP B-receptor mRNA in lung tissues. Total RNA from WKY (lanes 1 to 5) and SHR (lanes 6 to 10) were titrated with an increasing number (2×10^5 , 10^6 , 5×10^6 , 2×10^7 , 10^8) of molecules of mutated ANP A-receptor cRNA. Lanes 11 to 15 (WKY) and 16 to 20 (SHR) represent PCR transcript titration assay of ANP B-receptor mRNA with mutated ANP B-receptor cRNA as internal standard. (B) Typical titration curves of ANP A- and B-receptor mRNA in lungs from WKY and SHR. 2 μg of lung total RNA were titrated with increasing concentrations of mutated ANP A- or ANP B-receptor cRNA. Data points are plotted as % radioactivity in the upper (End.) or lower (Exo.) bands after EcoRI digestion vs. molecules of cRNA. The ANP A-receptor 50% titration point was 2.4×10^6 molecules for WKY and 8.4×10^6 molecules/ $2 \mu\text{g}$ total RNA for SHR. The 50% titration point of ANP B-receptor mRNA was 9.6

$\times 10^6$ molecules/ $2 \mu\text{g}$ total RNA for both WKY and SHR rats. (C) Histogram of ANP A- and ANP B-receptor mRNA levels per μg total RNA of lung tissues from WKY and SHR.

cyclase (A- and B-receptors) to C-receptors was not different between the SHR and WKY strains.

Using affinity cross-linking with ^{125}I -ANP in glomerular membranes, the density of radioactivity incorporated into 130- and 65-kD proteins was not clearly different between the two strains or between the ratio of 130–65-kD (C-receptor) proteins. In papillae also, where only the guanylate cyclase-related receptor (130-kD protein) was detectable, binding capacity in SHR was only slightly higher. These results indicate that the higher cGMP increase in response to ANP was not due to gross overexpression of total particulate guanylate cyclase (ANP A- and B-receptors) at the cell surface. We report in the present study a threefold higher ANP B-receptor mRNA level compared to ANP A-receptor mRNA in the rat lung. This presumably translates into a difference in cell surface receptor proteins. With this cross-linking method, a slight increase in the ANP A-receptor may stay undetected. The elevated cGMP levels observed in intact glomeruli from SHR could be due to the synthesizing enzyme itself (guanylate cyclase), to a decrease in cGMP metabolism by phosphodiesterase, or to a defect in its egression (75). We thus directly assessed the activity of guanylate cyclase *in vitro* in washed glomerular membranes and in various organs under control conditions, after ANP and Triton X-100 treatment, while inhibiting phosphodiesterase activity by MIX. A major, up to threefold difference was noted in the specific activity of the enzyme in glomerular membranes under basal conditions. The difference between the two strains decreased after ANP stimulation as well as under detergent-enhanced conditions. Thus, the enzyme's activity was higher in its natural membrane environment in SHR, and this characteristic was not compounded by greater sensitivity to ANP. These results suggest that either SHR guanylate cyclase was hyperactive in its basal state or that one guanylate cyclase population was increased but could not be detected by enhanced binding, indicating that ANP A-receptors expressed at a low level could be the target. In addition, the enzyme appeared to manifest a topographically different behavior. For example, in the renal papilla and zona glomerulosa, the difference in enzymatic activity observed under basal conditions was not found in the presence of the ligand or detergent. Conversely, the hyperactive state seen in SHR organs was not evident in the liver, pointing to the activation of one subtype of guanylate cyclase/ANP receptor.

Radiation-inactivation allows the *in situ* evaluation of molecular mass corresponding to a particular biological function. Only a few methods permit the study of protein size and interactions within their membranes without the necessity of prior solubilization or purification. Radiation-inactivation is one such technique. According to the target theory, in the simplest situations where only one component is responsible for the biological activity being assessed, radiation exposure leads to biological decay as a single exponential function of the absorbed dose (indicating destruction of crucial structures). The larger the structure, the more likely it will be hit. The only activity remaining after radiation exposure is due to units which have escaped ionization. They are fully active since there are no partially damaged targets. Our previous studies of the bovine adrenal cortex demonstrated that the ANP A-receptor is a multidomain protein. One domain of 20 kD represents the binding site of the ligand, whereas another domain of ~ 30 kD bears the guanylate cyclase function, the two being separated

by an inhibitory domain of 90 kD (88). Our results indicate that the protein expressed in SHR underwent no major structural change in the overall guanylate cyclase domain and in the region more specifically activated by the ligand. Moreover, the pattern of enzymatic decay after exposure to radiation was similar in normotensive and hypertensive animals, and no gross abnormality was uncovered in the size of the regulatory element controlling enzymatic activity. Subtle intramolecular anomalies having no impact on molecular mass or regulation of biological function would not be depicted by this method.

Quantitative PCR has been employed recently to accurately determine the absolute amount of low abundance, specific mRNA (89, 90). We constructed mutated ANP A- and B-receptor cDNAs for use as internal standards for the quantification of low levels of mRNA. Since many variables involved in reverse transcription and PCR can affect the yield of quantitative PCR, the use of identical standards varying in only two bases in the middle of the sequence is of great advantage. In this study, we found a significantly higher expression of ANP A-receptor mRNA in lungs from SHR compared to WKY rats (close to threefold). Expression of the B-receptor mRNA was similar between WKY and SHR. Further studies aimed at the determination whether differences in gene expression of ANP-A receptor are reflected at the protein level are currently being pursued in our laboratory.

In summary, in SHR, overexpression of the ANP A-receptor subtype could explain the overproduction of cGMP at basal levels. The abundance of the B-receptor over the A-receptor masked this overexpression in binding, cross-linking assays, and in total activation of guanylate cyclase by Triton X-100. Only precise mRNA quantitation of ANP receptor subtypes allowed the location of the difference between SHR and WKY.

It follows from our results that the abnormality in SHR should be observed only in tissues where the ANP A-receptor is expressed. In itself, this finding does not imply a primary pathogenetic role for this phenotype in the development of hypertension, but provides a tool to test this role in gene transfection and transgenic studies currently under way.

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