Characteristics of the renal C-type natriuretic peptide receptor in hypertrophied and developing rat kidney

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Abstract

This study investigates the effect of hypertrophy, using one kidney and one kidney/one clip rats, and development, comparing 3- and 12-week-old rats, on the expression of the 28-amino acid atrial natriuretic peptide (ANP₁₋₂₈) binding sites in rat kidney. Here we report an increased $B_{\max}$ value of glomerular binding sites for ANP₁₋₂₈ and C-type natriuretic peptide 1-22 (CNP₁₋₂₂) in hypertrophied and developing kidney, without modifying their affinity, an effect that was prevented in the presence of the synthetic des[Gln¹⁸, Ser¹⁹, Gly²⁰, Leu²¹, Gly²²]ANP₄₋₂₃-amide (C-ANF), suggesting that natriuretic peptide receptor (NPR)-C binding sites might be enhanced. The enhanced $B_{\max}$ was only detected in the high affinity binding site for CNP₁₋₂₂, which has been identified as the 67 kDa NPR-C-like protein. A similar effect was observed in renal glomeruli from 3-week-old rats compared with 12-week-old rats. Our results indicate that ANP₁₋₂₈, CNP₁₋₂₂ and C-ANF inhibited cAMP synthesis stimulated by the physiological agonists histamine and 5-hydroxytryptamine or directly by forskolin. The inhibitory effect was found to be significantly greater in 1-kidney and 1-kidney/1-clip rats than in controls, and in 3-week-old rats compared with 12-week-old rats. Our observations suggest that this effect must be attributed to the 67 kDa NPR-C-like protein due to the enhanced $B_{\max}$ values and the reported inhibitory role for this receptor on adenylyl cyclase activity. The enhanced inhibitory role of natriuretic peptides on cAMP synthesis in hypertrophied and developing kidney may influence glomerular function in the rat kidney and suggests a role for the 67 kDa NPR-C-like protein in growth.

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Introduction

Natriuretic peptides are physiological messengers involved in the regulation of a number of cellular functions. The 28-amino acid atrial natriuretic peptide (ANP₁₋₂₈) is involved in the homeostasis of body fluids and the cardiovascular system (Rademaker & Richards 2005). On the other hand, the function of other natriuretic peptides, such as brain, C-type or dendroaspis natriuretic peptides (BNP, CNP and DNP) is associated with renal and vascular homeostasis (Matsuo 2001, Dhingra *et al.* 2002, Woodard *et al.* 2002, Scotland *et al.* 2005). CNP has also been presented as a local modulator with antiproliferative effects in the vascular cell system (Matsuo 2001). The effects of these natriuretic peptides are mediated by three types of natriuretic peptide receptors (NPR), NPR-A, -B and -C (Nakao *et al.* 1992). NPR-A and NPR-B, of approximately 120 kDa, have agonist-dependent guanylate cyclase domains (Anand-Srivastava 1997, Woodard *et al.* 2004b, Fan *et al.* 2005). NPR-A is activated with high affinities by ANP₁₋₂₈ and BNP, but hardly binds CNP₁₋₂₂, which is known to be an agonist of NPR-B

(Koller *et al.* 1991). The third NPR, NPR-C, is a disulphide-bridged homodimer of 67 and 77 kDa units with a broad range of ligands, including ANP₁₋₂₈, BNP, CNP₁₋₂₂ and the synthetic des[Gln¹⁸, Ser¹⁹, Gly²⁰, Leu²¹, Gly²²]ANP₄₋₂₃-amide (C-ANF) (Koller *et al.* 1991), which shows no affinity for NPR-A or NPR-B (Koller *et al.* 1991, Brown & Chen 1995). The 77 kDa protein is involved in ligand internalization as a clearance receptor (Chen & Bennet 1998) and has a low affinity for CNP₁₋₂₂, while the 67 kDa protein shows a high affinity for CNP₁₋₂₂ and is involved in the regulation of fluid homeostasis by regulating cAMP synthesis (Anand-Srivastava & Trachte 1993, Woodard *et al.* 2004a).

A number of physiological effects mediated by ANP₁₋₂₈, such as the modulation of adrenergic neurotransmission, are associated with a reduction in the production of cAMP rather than with stimulation of cGMP synthesis. These effects are shared by the truncated analogue C-ANF, which, as well as ANP₁₋₂₈, inhibits the activity of adenylyl cyclase in some membrane preparations by a mechanism sensitive to guanosine triphosphate (GTP) and pertussis toxin (Anand-Srivastava & Trachte 1993).

A number of studies have reported that natriuretic peptides exert growth regulatory properties, and NPR-C has been presented as a candidate to mediate this function. Natriuretic peptides caused antimitogenic and antiproliferative effects in cultured glomerular mesangial cells in kidney (Appel 1990). Natriuretic peptides also showed growth-regulatory properties in a variety of other tissues and cells, such as brain, bone, myocytes, lung, red blood cell precursors and endothelial cells (Appel 1992, Lu *et al.* 2004, Murakami *et al.* 2004). Although initial data indicated that the antigrowth properties of natriuretic peptides correlated with the ability to generate cGMP (Appel 1990), it was shown that occupation of NPR-C by C-ANF blocks the antimitogenic effect of ANP_{1–28} on rat aortic smooth muscle cells (Cahill & Hassid 1994). In addition, it has been shown that ANP_{1–28} and C-ANF exert equipotent inhibitory effects of astrocyte proliferation, which suggests that NPR-C might account for these actions (Prins *et al.* 1996).

In the present study, we have investigated the possible relationship between the NPR-C-like proteins and tissue growth using experimental models of ‘one-kidney’ (1K) and ‘one-kidney/one-clip’ (1K/1C) rats, which induce kidney hypertrophy. In addition, we have performed the study in 3-week-old rats that are in a growth state. We have found that in hypertrophied and developing kidneys there is an increased glomerular expression of the 67 kDa NPR-C-like receptor. Consistent with this, we have observed that the ability of natriuretic peptides to inhibit agonist-induced cAMP production is increased under these conditions, which further supports the enhanced expression of the 67 kDa NPR-C-like protein and suggests a role for this receptor in the regulation of growth.

Materials and methods

Materials

¹²⁵I-Labelled ANP_{1–28}, ¹²⁵I-labelled CNP_{1–22}, ¹²⁵I standard, Hyperfilm ³H for autoradiography, and cAMP radioimmunoassay commercial kits were from Amersham Biosciences, UK. ANP_{1–28}, CNP_{1–22} and C-ANF were from Peninsula Laboratories (Merseyside, UK). Isobutylmethylxanthine (IBMX), histamine, 5-hydroxytryptamine (5-HT), forskolin, sodium dodecyl sulphate (SDS), bovine serum albumin (BSA), bromophenol blue, bicinchoninic acid protein assay reagent (BCA) and Tris were from Sigma (Poole, Dorset, UK). All other reagents were of analytical grade.

Animals

Rats were from Charles River (Margate, Kent, UK). Eighteen male Wistar-Kyoto rats (250–350 g, Charles

River Breeding) were separated into three equal groups: a control group, a one kidney group (1K) and a one kidney/one clip group (1K/1C). All procedures were approved by the Local Ethical Committee. 1K/1C rats were produced by partial constriction of the left renal artery with ligation and removal of the contralateral kidney under sodium pentobarbital anaesthesia (60 mg/kg intraperitoneally). 1K rats were subjected to right nephrectomy. Control rats were subjected to sham operation. All rats were maintained for 6 weeks on regular rat chow, pair-fed, and tap water was available *ad libitum*. Systolic blood pressure was measured indirectly by tail-cuff plethysmography (I.I.T.C., Life Science, USA), with the tail maintained at 37 °C. Six weeks after surgery, 18 rats from the 1K, 1K/1C and control groups were killed by decapitation. Kidneys were rapidly removed, placed in ice-cold Hank’s balanced salt solution (HBSS), containing (in mM): 137 NaCl, 10 HEPES, 5.4 KCl, 0.4 Mg₂SO₄, 0.34 Na₂HPO₄, 1.26 CaCl₂, 4.17 Na₂HCO₃, 0.44 K₂HPO₄, 0.49 MgCl₂, 0.2% (w/v) BSA and 5.56 glucose, pH 7.2, and weighed after death. Body weight was recorded immediately before decapitation.

Eighteen male 12-week-old Wistar-Kyoto rats (350–400 g) and eighteen male 3-week-old Wistar-Kyoto rats (81–92 g), maintained in an environment with constant humidity and temperature with free access to food and water, were killed by decapitation. Kidneys were rapidly removed, placed in HBSS and weighed after death. Body weight was recorded immediately before decapitation as described above.

Competitive inhibition of ¹²⁵I-ANP_{1–28} or ¹²⁵I-CNP_{1–22} binding

Consecutive 15 µm transverse sections through the level of the renal papilla from the kidneys of six rats in each group were suspended in PBS (containing in mM): 120 NaCl, 21.6 Na₂HPO₄, 8.4 NaH₂PO₄, pH 7.2, and supplemented with 1 mM 1,10-phenanthroline, a metalloproteinase inhibitor, which has been shown to inhibit the degradation of natriuretic peptides (Johnson & Foster 1990). Under our experimental conditions, HPLC studies have suggested that ¹²⁵I-ANP_{1–28} has a similar recovery pattern after incubation with renal sections from 1K, 1K/1C and control rats (64.7 ± 7.1, 66.9 ± 6.8 and 65.9 ± 6.4, *P* > 0.05) as well as from 3-week-old rats and 12-week-old rats (69.1 ± 8.5 and 67.8 ± 6.9, *P* > 0.05). This suggests that the effective concentrations of the radioligand were similar during incubations among renal sections from different group of rats.

Kidney slides were incubated with 100 pM ¹²⁵I-ANP_{1–28} or ¹²⁵I-CNP_{1–22} (2000 Ci/mmol) in the absence or presence of various concentrations (1 pM to 1 µM) of unlabelled rat ANP_{1–28}, CNP_{1–22}, or C-ANF

Table 1 Systolic blood pressure, heart weight, kidney weight and glomerular protein content of control, 1-kidney (1K), and 1-kidney, 1-clip (1K/1C) rats. Values are means $\pm$ S.E.M.

Variable	Control	1K rats	1K/1C rats
Blood pressure (mm Hg)	113 $\pm$ 8.7	121 $\pm$ 9.8	152 $\pm$ 7.8*
Body weight (g)	316 $\pm$ 11.2	309 $\pm$ 9.1	301 $\pm$ 8.7
Kidney weight (g)	0.93 $\pm$ 0.04†	1.34 $\pm$ 0.07*	1.29 $\pm$ 0.06
Heart weight (g)	1.03 $\pm$ 0.07	1.09 $\pm$ 0.10	1.19 $\pm$ 0.12
Protein content (μ g/glomeruli)	0.58 $\pm$ 0.05	0.60 $\pm$ 0.07	0.61 $\pm$ 0.09

* $P < 0.05$, for comparison of mean values between control and 1K, 1K/1C rats. †Weight of one kidney.

for 15 min at 20 °C as described previously (Woodard *et al.* 2005). After incubation, the sections were exposed to Hyperfilm ^3H for 31 days.

Protein content in tissue sections was determined by the Lowry protein assay. The values were used to convert B_{max} values from fmol/mm 2 to fmol/mg protein. Briefly, tissue blocks were washed and the cell suspension was then sonicated (Ultrasonics Inc. Model W-220F) in 1 ml 0.9% NaCl. Sample solution (50 μ l) or protein standard (50 μ l; range from 0–250 μ g protein/ml) was incubated with 1 ml BCA for 30 min at 60 °C. After incubation, the samples were read at 562 nm with a spectrophotometer (PU 8600 uv/vis, PYE UNICAM Ltd, UK). The linear relationship between the absorption at 562 nm and protein content was established for protein standards and this was used to obtain the protein content of unknown samples.

cAMP accumulation in renal glomeruli

Isolation of rat glomeruli was performed as described previously (Woodard *et al.* 2004a). Briefly, kidneys were removed and placed in ice-cold HBSS, containing (in mM): 137 NaCl, 10 HEPES, 5.4 KCl, 0.4 Mg $_2$ SO $_4$, 0.34 Na $_2$ HPO $_4$, 1.26 CaCl $_2$, 4.17 Na $_2$ HCO $_3$, 0.44 K $_2$ HPO $_4$, 0.49 MgCl $_2$, 0.2% (w/v) BSA and 5.56 glucose, pH 7.2. The cortices were minced and glomeruli were isolated by differential sieving. Aliquots of 100 glomeruli (7.6 mg protein) were suspended in HBSS plus 0.2% BSA with 1 mM IBMX and then incubated for 10 min at 20 °C with different agonists in the absence or presence of natriuretic peptides as indicated. Incubations were terminated by ice-cold trichloroacetic acid as previously described (Brown & Zuo 1992, 1994). Aliquots were then centrifuged at 4000 g for 10 min, and the supernatants were extracted with ether and radioimmunoassayed for cAMP as previously described (Brown & Zuo 1992, 1994).

Data analysis

The data on blood pressure, body weight and kidney weight are presented as means $\pm$ S.E.M. Data were

analysed using the LIGAND program. Analysis of statistical significance was performed using the Student's *t*-test. The significance level was $P < 0.05$.

Results

Competitive inhibition of ^{125}I -ANP $_{1-28}$ binding in hypertrophied rat kidneys

In order to investigate the properties of NPR in hypertrophied kidneys, we used the 1K and the 1K/1C rat models. Table 1 summarizes the systolic blood pressure, heart weight, kidney weight and glomerular protein content of 1K, 1K/1C and control rats. As shown in Table 1, the kidney weight of both 1K and 1K/1C rats is increased in comparison with that of control rats ($P < 0.05$). In addition, blood pressure in 1K/1C rats is significantly elevated compared with control rats ($P < 0.01$).

As shown in Fig. 1, autoradiographs revealed specifically reversible binding of 100 pM ^{125}I -ANP $_{1-28}$ mainly to glomeruli of control, 1K and 1K/1C rats. The competitive inhibition of ^{125}I -ANP $_{1-28}$ by unlabelled ANP $_{1-28}$ on glomeruli of control, 1K and 1K/1C rats was consistent with reversible binding sites for ANP $_{1-28}$. The pKa values are not significantly different among the three groups of rats, but the B_{max} values from 1K and 1K/1C rats are significantly higher than those from the control rats (Table 2; $P < 0.01$), which indicated that receptor binding to ANP $_{1-28}$ is significantly increased in the glomeruli of both 1K and 1K/1C rats. ANP $_{1-28}$ binding to the papilla is not significantly different among these three group of rats ($P > 0.05$). The pKa and B_{max} were, respectively, 8.31 ± 1.3 and 729 ± 89 fmol/mg protein for control rats, 8.46 ± 1.7 and 819 ± 91 fmol/mg protein for 1K rats and 8.18 ± 1.4 and 799 ± 83 fmol/mg protein for 1K/1C rats ($n = 6$). The papilla expresses NPR-A but not detectable NPR-C-like binding sites in the rat (Brown & Zuo 1992). Consistent with this, 1 μ M C-ANF did not significantly alter the specific binding of 100 pM ^{125}I -ANP $_{1-28}$ to the papilla in control rats ($91.1 \pm 7.7\%$ of control), 1K rats ($89.1 \pm 9.7\%$ of control) or 1K/1C rats ($91.3 \pm 9.1\%$ of

Figure 1 Autoradiographs of renal binding of 100 pM ¹²⁵I-ANP_{1–28} in rats. Sections of kidney from control, 1K rats and 1K/1C rats were incubated with 100 pM ¹²⁵I-ANP_{1–28} in the absence (A, B and C) and presence of 1 μM ANP_{1–28} (D, E and F), CNP_{1–22} (G, H and I) and C-ANF (J, K and L) at 20 °C for 15 min. Autoradiography was performed as described under Materials and methods. The autoradiographs are representative of six separate determinations.

Table 2 Binding constants for the specifically reversible binding of ANP_{1–28} or CNP_{1–22} in the glomeruli of control, 1 kidney (1K), and 1 kidney, 1 clip (1K/1C) rats. Values are means±S.E.M.

	Control rats			1K rats			1K/1C rats		
	pKa	K _d (nM)	B _{max} (fmol/mg protein)	pKa	K _d (nM)	B _{max} (fmol/mg protein)	pKa	K _d (nM)	B _{max} (fmol/mg protein)
Ligand									
HA+A	8.39±1.01	4.16	555±78	8.52±0.81	3.00	929±84**	8.43±1.30	3.78	902±38**
HA+C	8.52±0.33	2.44	358±14	8.55±0.94	2.44	853±37**	8.46±0.85	2.44	669±14**
	5.62±0.90	3.48	138±11	3.82±1.21	2.82	141±7	3.42±1.21	3.91	144±7
HC+C	8.31±1.20	4.97	226±33	8.26±1.00	5.50	783±97**	8.14±0.83	7.32	685±79**
HC+A	8.07±0.73	8.52	267±9.1	8.25±0.73	5.58	765±35**	8.25±1.20	5.56	771±49**

The maximum binding capacities (B_{max}) and pKa were assessed from the competitive inhibition of the binding of 100 pM ¹²⁵I-ANP_{1–28} and 200 pM ¹²⁵I-CNP_{1–22} by various concentrations of unlabelled ANP_{1–28} or CNP_{1–22}. Apparent dissociation constants (K_d) are also given. **P<0.01 relative to control rats. HA, ¹²⁵I-ANP_{1–28}; A, ANP_{1–28}; HC, ¹²⁵I-CNP_{1–22}; C, CNP_{1–22}. In HA+C data for high and low affinity binding sites are reported.

Figure 2 Autoradiographs of renal binding of 100 pM ^{125}I -CNP $_{1-22}$ in rats. Sections of kidney from control, 1K rats and 1K/1C rats were incubated with 100 pM ^{125}I -CNP $_{1-22}$ in the absence (A, B and C) and presence of 1 μM CNP $_{1-22}$ (D, E and F), ANP $_{1-28}$ (G, H and I) and C-ANF (J, K and L) at 20 °C for 15 min. Autoradiography was performed as described under Materials and methods. The autoradiographs are representative of six separate determinations.

control). On the other hand, in renal glomeruli the competitive inhibition of ^{125}I -ANP $_{1-28}$ by unlabelled C-ANF showed that 1 μM C-ANF abolished the difference of ANP $_{1-28}$ binding among the three groups. Thus, the B_{max} values for specific glomerular binding of ^{125}I -ANP $_{1-28}$ in the presence of C-ANF were 159.6 ± 12.3 , 169.6 ± 21.3 and 157.6 ± 18.1 fmol/mg protein in control, 1K and 1K/1C rats ($P > 0.05$). This suggests that the expression of the NPR-C-like binding sites on glomeruli increases in 1K and 1K/1C rats, but NPR-A remains unaltered in these two groups compared with controls.

The competitive inhibition of ^{125}I -ANP $_{1-28}$ binding to renal glomeruli by CNP $_{1-22}$ confirmed this conclusion by showing two binding sites with different affinities for CNP $_{1-22}$ in control, 1K and 1K/1C rats. The pKa and B_{max} values for these two binding sites are shown in Table 2. Our results indicate that the binding site with high affinity for CNP $_{1-22}$, which resembles the 67 kDa

protein, was significantly increased in the glomeruli of both 1K and 1K/1C rats compared with controls ($P < 0.01$); however the binding site with low affinity for CNP $_{1-22}$, which resembles the 77 kDa protein, is not altered in 1K/1C rats ($P > 0.05$).

Competitive inhibition of ^{125}I -CNP $_{1-22}$ binding in hypertrophied rat kidneys

As shown in Fig. 2, autoradiographs revealed that 100 pM ^{125}I -CNP $_{1-22}$ binds to glomeruli of the three groups of rats. The binding was restricted to glomeruli in the three experimental groups and was reversibly inhibited by increasing concentrations of unlabelled CNP $_{1-22}$. This inhibition was consistent with glomerular binding sites of uniform affinity for this ligand. The pKa and B_{max} values for the glomerular site among the three groups of rats are shown in Table 2. The pKa values were

not significantly different ($P>0.05$), which suggests that the characteristic ligand affinities of these NPR-C-like sites have no significant difference among the three groups of rats. However, the $B_{\max}$ values indicated that this class of binding was significantly increased in 1K and 1K/1C rats compared with the control group ($P<0.01$). Further competitive inhibition of ^{125}I -CNP₁₋₂₂ binding to glomeruli by ANP₁₋₂₈ revealed that this class in each group had high affinity for CNP₁₋₂₂ and ANP₁₋₂₈ (Table 2). The increase in glomerular $B_{\max}$ for natriuretic peptides found with ^{125}I -CNP₁₋₂₂ was similar to the increase in glomerular $B_{\max}$ for ANP₁₋₂₈ discovered with ^{125}I -ANP₁₋₂₈ as well as to the increase in $B_{\max}$ for the class of glomerular sites with high affinity for CNP₁₋₂₂ revealed by ^{125}I -ANP₁₋₂₈. All the binding data, therefore, support the idea that kidney hypertrophy in 1K and 1K/1C rats significantly increases the expression of the high affinity NPR-C-like protein binding site without changing its affinity to the ligand or altering sites related to NPR-A or to the low affinity NPR-C-like protein.

Effects of ANP₁₋₂₈, CNP₁₋₂₂ or C-ANF on cAMP stimulated by histamine, 5-hydroxytryptamine or forskolin

The basal rate of glomerular cAMP accumulation in the presence of IBMX was 2.5 ± 0.3 , 2.0 ± 0.3 and 2.1 ± 0.4 fmol/mg protein in control, 1K and 1K/1C rats respectively. Treatment of renal glomeruli from control rats with the physiological agonists histamine and 5-hydroxytryptamine (5-HT) or with forskolin significantly increased the glomerular cAMP production in control rats in a concentration dependent manner (Fig. 3; $n=6$). We have found that treatment of renal glomeruli with ANP₁₋₂₈, CNP₁₋₂₂ and C-ANF reduced cAMP production stimulated by 5 μM histamine, 0.1 μM 5-HT and 10 μM forskolin (Fig. 4; $n=6$). These effects were significantly greater in glomeruli from 1K and in most cases was also greater in 1K/1C rats (Fig. 4; $P<0.05$). These findings suggest that kidney hypertrophy increases the glomerular expression of the 67 kDa NPR-C-like protein.

Competitive inhibition of ^{125}I -ANP₁₋₂₈ and ^{125}I -CNP₁₋₂₂ binding in 3- and 12-week-old rats

Comparison of autoradiography with the corresponding stained tissue sections revealed a specific reversible

Figure 3 Concentration-dependent cAMP generation by agonists in glomeruli from control rats. Glomeruli from control rats were stimulated with increasing concentrations of histamine (1–10 μM; A), 5-hydroxytryptamine (5-HT; 0.01–0.5 μM; B) or forskolin (5–50 μM; C). cAMP synthesis was measured as described under Materials and methods; values are expressed as fmol/mg protein and are presented as means $\pm$ S.E.M. from six separate experiments.

Figure 4 Effect of natriuretic peptides on cAMP generation in glomeruli from control, 1K and 1K/1C rats. Glomeruli from control, 1K and 1K/1C rats were stimulated with 5 μ M histamine, 0.1 μ M 5-hydroxytryptamine (5-HT) or 10 μ M forskolin in the absence and presence of ANP₁₋₂₈ (A), CNP₁₋₂₂ (B) or C-ANF (C). cAMP synthesis was measured as described under Materials and methods; values are expressed as percentage inhibition of the response in the absence of natriuretic peptides and are presented as means $\pm$ S.E.M. from six separate experiments. * $P < 0.05$ compared with the effect observed in control rats.

binding site for ANP in 3- and 12-week-old rats (Fig. 5). 125 I-ANP₁₋₂₈ and 125 I-CNP₁₋₂₂ bound mostly to glomeruli. Radioligand binding to these structures was virtually abolished in the presence of 1 μ M unlabelled ANP₁₋₂₈, CNP₁₋₂₂ and C-ANF (Fig. 5; $n=6$).

Competitive inhibition of 125 I-ANP₁₋₂₈ binding by ANP₁₋₂₈ and CNP₁₋₂₂ was further examined in 3- and 12-week-old rats. ANP₁₋₂₈ displaced 125 I-ANP₁₋₂₈ in a concentration-dependent manner both in 3- and 12-week-old rats (data not shown). We found that 3-week-old rats have a greater $B_{\max}$ value of the glomerular binding sites for ANP₁₋₂₈ but a similar affinity for this peptide (Table 3). A similar effect was observed when 125 I-CNP₁₋₂₂ binding was tested. In 3-week-old rats the $B_{\max}$ value of the glomerular binding sites for CNP₁₋₂₂ was increased without any detectable effect on its affinity (Table 3). Further competitive inhibition of 125 I-CNP₁₋₂₂ binding to glomeruli by ANP₁₋₂₈ and C-ANF revealed that this class has high affinity for CNP₁₋₂₂, ANP₁₋₂₈ and C-ANF (not shown).

These results indicate that the NPR-C-like binding site, which resembles the 67 kDa protein, significantly increases in the developing kidney of 3-week-old rats, while the binding sites related to NPR-A-like or NPR-C-like binding sites resembling the 77 kDa protein remained unaltered.

The competitive inhibition of the binding of 125 I-ANP₁₋₂₈ to glomeruli by CNP₁₋₂₂ showed two binding sites with different affinities for CNP₁₋₂₂ in 12- and 3-week-old rats. The pK_a and $B_{\max}$ for these two binding sites are shown in Table 3 and suggest that the binding site with high affinity for CNP₁₋₂₂ was significantly increased in 3- compared with 12-week-old rats ($P < 0.01$); in contrast, the binding site with low affinity for CNP₁₋₂₂ remained unchanged in both 3- and 12-week-old rats ($P > 0.05$).

Meanwhile, the competitive inhibition of 125 I-ANP₁₋₂₈ by unlabelled C-ANF on glomeruli of the two groups of rats showed that 1 μ M C-ANF abolished the binding differences between them (not shown). The

Figure 5 Autoradiographs of renal binding of 100 pM ^{125}I -ANP₁₋₂₈ or ^{125}I -CNP₁₋₂₂ in 3- and 12-week-old rats. Sections of kidney from 3- and 12-week-old rats were incubated with 100 pM ^{125}I -ANP₁₋₂₈ or ^{125}I -CNP₁₋₂₂ in the absence and presence of 1 μM ANP₁₋₂₈, CNP₁₋₂₂ and C-ANF, as indicated, at 20 °C for 15 min. Autoradiography was performed as described under Materials and methods.

specific glomerular binding of ^{125}I -ANP₁₋₂₈ in the absence of C-ANF was 467 ± 73 fmol/mg protein in 12-week-old rats and 909 ± 132 fmol/mg protein in

3-week-old rats ($P < 0.01$), while the corresponding figures with C-ANF were 167 ± 58 fmol/mg protein and 164 ± 71 fmol/mg protein ($P > 0.05$). These results

Table 3 Binding constants for the specifically reversible binding of ANP₁₋₂₈ or CNP₁₋₂₂ in the glomeruli of 3- and 12-week old rats. Values are means $\pm$ S.E.M.

	3-week-old rats			12-week-old rats		
	pKa	K_d (nM)	B_{max} (fmol/mg protein)	pKa	K_d (nM)	B_{max} (fmol/mg protein)
Ligand						
HA+A	8.48 ± 0.51	3.31	$909 \pm 32^{**}$	8.46 ± 0.20	3.40	467 ± 13
HA+C	8.46 ± 0.85	2.40	$769 \pm 14^{**}$	8.52 ± 0.33	2.44	358 ± 14
	3.42 ± 1.21	3.93	144 ± 7.4	5.62 ± 0.93	3.48	238 ± 11
HC+C	8.54 ± 0.74	2.90	$534 \pm 36^{**}$	8.44 ± 1.10	3.71	263 ± 27

The maximum binding capacities (B_{max}) and pKa were assessed from the competitive inhibition of the binding of 100 pM ^{125}I -ANP₁₋₂₈ and 200 pM ^{125}I -CNP₁₋₂₂ by various concentrations of unlabelled ANP₁₋₂₈ or CNP₁₋₂₂. Apparent dissociation constants (K_d) are also given. $^{**}P < 0.01$ relative to 12-week old rats. HA, ^{125}I -ANP₁₋₂₈; A, ANP₁₋₂₈; HC, ^{125}I -CNP₁₋₂₂; C, CNP₁₋₂₂. In HA+C data for high and low affinity binding sites are reported.

Figure 6 Effect of natriuretic peptides on cAMP generation in glomeruli from 3- and 12-week-old rats. Glomeruli from 3- and 12-week-old rats were stimulated with 5 μ M histamine, 0.1 μ M 5-hydroxytryptamine (5-HT) or 10 μ M forskolin in the absence and presence of ANP₁₋₂₈ (A), CNP₁₋₂₂ (B) or C-ANF (C). cAMP synthesis was measured as described under Materials and methods; values are expressed as percentage inhibition of the response in the absence of natriuretic peptides and are presented as means $\pm$ S.E.M. from six separate experiments. * $P < 0.05$ compared with the effect observed in control rats.

suggest that NPR-C-like binding sites increase in 3-week-old rats compared with the 12-week-old rats while the NPR-A binding site remains unchanged.

Effects of ANP₁₋₂₈, CNP₁₋₂₂ or C-ANF on cAMP stimulated by histamine, 5-HT or forskolin in 3- and 12-week-old rats

The basal rate of glomerular cAMP accumulation in the presence of IBMX was 3.1 ± 0.2 and 3.0 ± 0.2 fmol/mg protein in 3- and 12-week-old rats. Histamine (5 μ M), 5-HT (0.1 μ M) and forskolin (10 μ M) increased cAMP content in both groups of rats. As shown in Fig. 6, treatment with 1 μ M ANP₁₋₂₈, CNP₁₋₂₂ or C-ANF reduced cAMP production by these agents significantly ($P < 0.05$; $n = 6$). However, the inhibitory effect of the natriuretic peptides was significantly higher in 3-week-old rats compared with 12-week-old rats (Fig. 6; $P < 0.05$).

Discussion

Our results indicate that kidney hypertrophy increases the expression of the 67 kDa NPR-C-like protein in renal glomeruli. As a result, ANP₁₋₂₈, CNP₁₋₂₂ and C-ANF show a greater inhibitory effect on cAMP production stimulated by the agonists histamine, 5-HT or forskolin in glomeruli from hypertrophied kidney rats as compared with those from controls.

The autoradiographic study agrees with previous results reporting that rat renal glomeruli express two types of NPR-C-like receptors (Woodard *et al.* 2004a). The competitive inhibition of ¹²⁵I-ANP₁₋₂₈ binding to the glomeruli by CNP₁₋₂₂ showed that there were two binding sites with different affinities for CNP₁₋₂₂, one with high affinity, supposedly the 67 kDa NPR-C-like protein, and the other with low affinity, the 77 kDa NPR-C-like receptor.

Our results indicate that expression of NPR-C-like binding sites was increased in 1K and 1K/1C rats as

well as in 3-week-old rats, but that NPR-A and NPR-B remained unaltered. We further showed that the NPR-C-like protein with high affinity for CNP_{1-22} , described as the 67 kDa NPR-C-like binding site (Anand-Srivastava & Trachte 1993), rather than that with low affinity, identified as the 77 kDa NPR-C-like protein (Anand-Srivastava & Trachte 1993), is the binding site that is increased in the experimental groups mentioned above. Although the different B_{max} values in NPR-C between 1K, 1K/1C and control rats, as well as between 3- and 12-week-old rats, may be attributed to the differences in radioligand breakdown during incubation with renal sections, HPLC studies have reported that a similar amount of ^{125}I -ANP $_{1-28}$ was recovered after incubation with renal sections from 1K, 1K/1C and control rats, as well as from 3- and 12-week-old rats, suggesting that the effective concentrations of radioligand were similar for the different experimental groups. In addition, the renal sections were preincubated and washed in 150 mM sodium chloride plus 0.5% acetic acid (pH 5.0) at 4 °C for 10 min to completely remove the endogenous bound ligands (Gunning *et al.* 1988, Martin & Lewicki 1989). Therefore, the differences in the B_{max} values among the different groups of rats could not be attributed to the differences in endogenous bound ligands.

cAMP production stimulated by the agonists histamine, 5-HT or forskolin were examined in the presence of IBMX, an inhibitor of phosphodiesterases that inhibits the cGMP-activated phosphodiesterase that may account for the effects of natriuretic peptides on cAMP levels (Beavo & Reifsnyder 1990). Therefore, our findings suggest that natriuretic peptides induce a greater reduction of cAMP levels in glomeruli from 1K and 1K/1C rats by decreasing the rate of cAMP synthesis more effectively in these groups. In addition, our results indicate that natriuretic peptides induce a greater inhibition of cAMP production stimulated by physiological agonists, such as histamine or 5-hydroxytryptamine, in developing 3-week-old rats than in 12-week-old rats, which further suggests the physiological significance of natriuretic peptides.

The 67 kDa NPR-C-like protein with high affinity for CNP_{1-22} has been reported to inhibit cAMP synthesis (Brown & Zuo 1994, Woodard *et al.* 2004a). The increase in the B_{max} value for the high affinity binding site of NPR-C in both the hypertrophied and developing kidney groups of rats together with the increase in the ability to inhibit cAMP generation confirms the view that the enhanced NPR-C-like binding site is the 67 kDa NPR-C-like protein and may be involved in the regulation of cAMP levels.

There is still some controversy at present about the mechanism by which natriuretic peptides inhibit cAMP production after binding to the 67 kDa NPR-C-like receptor. ANP $_{1-28}$ is known to decrease cAMP levels in

some tissues by stimulating the synthesis of cGMP, which, in turn, activates a cGMP-dependent phosphodiesterase of cAMP (McFarland *et al.* 1991). On the other hand, a number of studies have suggested that CNP_{1-22} , BNP and C-ANF interact with NPR-C receptors resulting in the inhibition of adenylyl cyclase activity probably by the activation of a pertussis-sensitive G_i protein (Anand-Srivastava 1997, Rose *et al.* 2003, Callahan *et al.* 2004, Woodard *et al.* 2004a). Our results did not support the concept that natriuretic peptides decrease cAMP via a cGMP-dependent phosphodiesterase since CNP_{1-22} -induced inhibition of cAMP production was detected at a concentration of 1 nM which is 100-fold lower than the minimum dose required to stimulate cGMP production in rat glomeruli (data not shown). Moreover, the actions of natriuretic peptides on cAMP production were investigated in the presence of 1 mM IBMX in our conditions. These findings indicate that natriuretic peptides reduced cAMP synthesis by inhibiting the rate of cAMP generation rather than by activating a cGMP-dependent phosphodiesterase specific for cAMP.

The fact that the expression of 67 kDa NPR-C-like protein significantly increases in 1K and 1K/1C rats as well as in 3-week-old rats suggests that this receptor may be involved in growth regulation, since the kidney in both 1K and 1K/1C rats shows significant hypertrophy and that of 3-week-old rats is in a developing state. Recent evidence supports the view that natriuretic peptides have growth-regulatory properties; however, the cellular mechanisms involved in this role are not well established, although NPR-C may be involved in this function (Cahill & Hassid 1991, Lelievre *et al.* 2001). A recent study reports that epidermal growth factor reduces the expression of NPR-C by a mechanism involving activator protein-1, which suggests that NPR-C might mediate the growth-regulatory actions of ANP (Placier *et al.* 2001). Therefore, it has been suggested that NPR-C is involved in the regulation of cell growth; however, it remains to be elucidated which type of NPR-C-like protein is involved in this function. The increase in expression of the 67 kDa NPR-C-like protein in rats with developing and hypertrophied kidneys suggests that this receptor might be involved in the regulation of tissue growth. Although speculative, the 67 kDa NPR-C-like receptor may be involved in the regulation of growth through the inhibition of cAMP synthesis as there is a body of evidence supporting a role for cyclic nucleotides in cell proliferation in a variety of tissues (Dremier *et al.* 2002, Dormond & Ruegg 2003), including kidney (Yamaguchi *et al.* 2000, Ito *et al.* 2004).

Although several studies reported that both NPR-A and NPR-C are down regulated in 1K/1C rats compared with normotensive 1K rats as a result of hypertension, we have not found significant differences in the expression of NPR-C between 1K and 1K/1C

rats under our experimental conditions. One reason for this discrepancy might be the different rat strain used, WKY rats in our study and Sprague-Dawley rats in others (Bonhomme & García 1993). Alternatively, we have found that the amount of kidney hypertrophy in 1K and 1K/1C rats is similar under our conditions, while in other studies, the kidneys in 1K/1C rats showed significantly less hypertrophy than those in 1K rats (García *et al.* 1988).

In summary, our observations demonstrate that two types of NPR-C-like proteins that resemble the 67 kDa and 77 kDa proteins are independently regulated receptors. Expression of the receptor resembling the 67 kDa protein is significantly increased in renal glomeruli under hypertrophy and growth situations. In addition, natriuretic peptides have an enhanced inhibitory effect on cAMP synthesis in hypertrophied and developing kidney, which may influence glomerular function, suggesting a role for the 67 kDa NPR-C-like protein in the physiological regulation of tissue growth through the modulation of cAMP synthesis.

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