Modulation of endocardial natriuretic peptide receptors in right ventricular hypertrophy

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Kim, Sung Zoo, Kyung Woo Cho, and Suhn Hee Kim. Modulation of endocardial natriuretic peptide receptors in right ventricular hypertrophy. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H2280–H2289, 1999.—Natriuretic peptide (NP) receptors (NPRs) located at the endocardial endothelium are suggested to be involved in regulating myocardial contractility. However, the characteristics and modulation of NPRs in relation to cardiac failure are not well defined. This study examined the properties of NPRs in ventricular endocardium using quantitative receptor autoradiography, RT-PCR, Southern blot analysis, and activation of particulate guanylyl cyclase (GC) by NPs. In control rats, specific[^125I]-labeled rat atrial NP (rANP)(1–28) binding sites were localized in right (RV) and left ventricular (LV) endocardium. Binding affinities of[^125I]-rANP(1–28) were remarkably higher in RV than LV endocardium. Radioligand binding at these sites was mostly inhibited by des[^Gln18, Ser19, Gly20, Leu21, Gly22]-ANP(4–23), a specific NP clearance receptor ligand. mRNAs for all three recognized NPRs were detected in endocardial cells by RT-PCR and confirmed by Southern blot analysis. Production of cGMP by particulate GC in endocardial cell membranes was stimulated by NPs with a rank order of potency of C-type NP(1–22) >> brain NP (BNP)(1–26) > ANP(1–28). We also examined the modulation of these NPRs during cardiac hypertrophy induced by monocrotaline (MCT). In MCT-treated rats with pulmonary hypertension, specific[^125I]-rANP(1–28) binding to hypertrophied RV endocardium almost disappeared and cGMP production by NPs was significantly decreased. In rats with pulmonary hypertension, plasma levels of ANP and BNP were increased by fivefold compared with controls. The results indicate that there is a differential distribution of NPRs in the cardiac chambers, with the most abundant binding sites in RV endocardium, that NPR-B is the predominant GC-coupled NPR in ventricular endocardium, and that endocardial NPRs are downregulated with ventricular hypertrophy. Downregulation of NPRs may be associated with an increment of endogenous NP production caused by mechanical overload in hypertrophied ventricle.

atrial natriuretic peptide; brain natriuretic peptide; receptor autoradiography; reverse transcriptase-polymerase chain reaction; Southern blot; guanosine 3′,5′-cyclic monophosphate; ventricular endocardium; monocrotaline

ATRIAL NATRIURETIC PEPTIDE (ANP), a 28-amino acid hormone, was originally identified in the heart (17, 21) and is primarily synthesized in the atrial myocytes and released into the bloodstream. Both mechanical stretch and the rate of atrial contraction are well-known direct stimuli of ANP synthesis and release (12, 14, 18, 38). Previous studies on various cardiac disorders with associated changes in plasma levels of ANP (9, 45) contributed to our understanding of the regulation of body fluids, electrolytes, and blood pressure homeostasis by ANP in pathological states. This peptide exerts cardiovascular and renal effects via activation of particulate guanylyl cyclase (GC)-coupled specific receptors (22). Since C-type natriuretic peptide (CNP) was isolated from porcine brain (42), natriuretic peptide receptors (NPRs) have been classified in three subtypes (NPR-A, NPR-B, and NPR-C). NPR-A and NPR-B exhibit intrinsic GC activity and mediate many of the well-known biological functions of natriuretic peptides (NPs). NPR-C (clearance receptor subtype) has no GC activity but binds and internalizes all kinds of endogenous NPs for clearance (11, 29, 32).

Three NPR genes are expressed in ventricular as well as atrial tissues (7, 37, 44). Furthermore, transcripts for all three NPR subtypes have been found in atrial and ventricular cardiomyocytes (31). However, NPR seems to be more prominent in the endocardium than in the myocardium of the cardiac ventricles (4, 39).

Several groups have studied the modulation of ANP synthesis and release during cardiac hypertrophy induced by experimental pulmonary hypertension (1, 2, 16, 25). Monocrotaline (MCT)-induced pulmonary hypertension is one of the paradigms used to cause right ventricular hypertrophy (27, 35). In this model, it was reported that the levels of ANP in plasma and right ventricle and of ANP mRNA in right ventricle were higher in MCT-treated rats than in control rats (2, 16). NPR-C transcripts of all cardiac chambers were shown to disappear in rats with aortovenocaval fistula (7). Although it was suspected that the characteristics of NPRs are modulated differently in the hemodynamically different cardiac chambers, their modulation with cardiac hypertrophy caused by the fistula was not fully characterized. Therefore, we have performed experiments to characterize receptor binding properties, NPR gene expression, and cGMP production in the ventricular endocardium of control rats and their modulation in the hypertrophied right ventricle of MCT-induced pulmonary hypertensive rats.

METHODS

Animals. For production of right ventricular hypertrophy, male Sprague-Dawley rats weighing 220–250 g were given a single subcutaneous injection of 60 mg/kg of MCT (Sigma Chemical, St. Louis, MO) in the interscapular region. MCT was dissolved in 1 N HCl, neutralized with 1 N NaOH, and then diluted to a 2% solution with sterile distilled water. Control rats were injected with the same volume of vehicle.

[^125I] RITUXIMAB: A Novel Monoclonal Antibody for the Treatment of Chronic Lymphocytic Leukemia

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All animals had a free supply of food and water until they were killed.

Tissue preparation. Four weeks after the MCT and vehicle injections, the animals were killed by decapitation. For the determination of the concentration of plasma ANP and brain natriuretic peptide (BNP), arterial blood samples were collected into prechilled tubes containing EDTA (1 mg/ml of whole blood), phenylmethylsulfonyl fluoride (PMSF, 0.4 mg/ml), and soybean trypsin inhibitor (50 µg of benzoyl-L-arginine ethyl ester U/ml). Plasma samples were obtained after centrifugation at 10,000 g for 15 min at 4°C. For in vitro receptor autoradiography, the heart was rapidly removed and snap frozen in liquid nitrogen. Sections (20 µm) with transverse plane of right and left ventricles were cut in a cryostat at −20°C, thaw mounted on gelatin-chrom-alum-coated slides, and then dried in a desiccator overnight at 4°C.

Iodination of rat ANP (1–28). 125I-labeled rat ANP (1–28) [125I-rANP(1–28)] was prepared as described previously (12). Synthetic rANP(1–28) (Peninsula Laboratories, Belmont, CA) and 2,4-dinitrophenylated Na (Amersham International, Amersham, UK) were used for iodination by the chloramine T method. The iodinated rANP (1–28) was divided and stored at −70°C until used. The specific activity (~2,000 Ci/mmol) of 125I-rANP (1–28) was determined by RIA (12). For RIA of ANP (1–28), antibody was prepared from New Zealand White rabbits using a method described previously (13).

Determination of plasma ANP and BNP concentrations. Plasma levels of ANP were measured by RIA after extraction using Sep-Pak C18 cartridges (Waters Associates) as previously described (13, 14).

Plasma BNP was also extracted using Sep-Pak C18 cartridges by the same procedures as those for ANP extraction. The recovery rate of BNP was 68.3 ± 0.9%. Plasma levels of BNP were measured by RIA as follows. The lyophilized samples were reconstituted with 100 mM phosphate buffer (pH 7.4) containing 50 mM NaCl, 0.1% BSA, 0.1% Triton X-100, and 0.01% sodium azide. After incubation with anti-rBNP-32 antibody (Phoenix Pharmaceutical, Mountain View, CA) for 24 h at 4°C, −12,000 counts/min (cpm) of 125I-labeled rat BNP-32 (specific activity 1,828 Ci/mmol, Peninsula Laboratories) were added to each reaction mixture and incubated for another 24 h at 4°C. The separation of the free from the bound form was achieved by the addition of second antibody, and the radioactive pellet was counted in a gamma counter (Packard Instrument, Meriden, CT). RIA for BNP was done on the day of experiments, and all samples in an experiment were analyzed in a single assay. Nonspecific binding was <1.5%. The 50% intercept was at 29.9 ± 1.7 pg/tube (n = 6). The intra- and interassay coefficients of variation were 2.4% (n = 10) and 9.1 (n = 4) %, respectively.

In vitro autoradiographic binding of 125I-rANP (1–28). The conditions for binding of 125I-rANP (1–28) to the heart section with transverse plane of right and left ventricles were prepared according to a method described elsewhere (5, 28).

Briefly, the sections were washed with 150 mM NaCl-0.5% acetic acid (pH 5.0) at room temperature for 10 min to remove endogenous ANP and then preincubated with 30 mM phosphate buffer (pH 7.2) containing 120 mM NaCl and 1 mM phenanthroline at room temperature for 8 min. They were then incubated with 250 pM 125I-rANP (1–28) in fresh preincubation buffer containing 40 µM/ml bacitracin, 100 µg/ml PMSF, 10 µg/ml leupeptin, and 0.5% BSA at room temperature for 60 min.

To characterize NPR subtypes, the competitive inhibition of 125I-rANP (1–28) binding of the ventricular endocardium was examined in consecutive sections from six rats of each group by coincubating with various concentrations (1 pM to 1 µM or 10 µM) of unlabeled rANP (1–28) or des[Gln18, Ser19, Gly20, Leu21, Gly22] ANP (1–23) (C-ANP, Peninsula Laboratories) as specific ligands for the NPR-C subtype. To test the specificity of the binding of radioligands, adjacent sections were incubated in the presence of the unrelated peptides angiotensin II or arginine vasopressin (both 10 µM, Peninsula Laboratories). The effects of prior receptor occupancy were examined in sections from the kidneys of other control rats and MCT-treated rats with right ventricular hypertrophy. Sections from the hearts from each group were incubated with and without 150 mM NaCl-0.5% acetic acid (pH 5.0) for 10 min at room temperature. They were then washed in four changes of preincubation buffer, each for 2 min at room temperature, before being incubated with 250 pM 125I-rANP (1–28) in the presence or absence of 1 µM unlabeled rANP (1–28).

After incubation, the sections were rinsed and washed with fresh preincubation buffer for 5 min at 4°C. Subsequently, they were rinsed three times in cold distilled water at 4°C and quickly dried under a stream of cold air.

Quantitative analysis of autoradiograms. Autoradiographic images were generated by exposing sections to Hyperfilm–H (Amersham International) in X-ray cassettes together with 125I-prelabeled polymer standard strips (Amersham International) at room temperature for 7 days. Autoradiograms were developed in Kodak D-19 (Eastman Kodak, Rochester, NY) for 3 min and fixed in Kodak rapid fixer for 5 min at room temperature. The precise localization of specific 125I-rANP (1–28) binding and any cluster at the edge of ventricular wall and septal side in the heart sections was confirmed by counterstaining with hematoxylin and eosin under microscopy (5, 28).

Autoradiographic images were viewed with a Leica Wild M420 Microscope and captured using a Sony video camera with a charge-coupled device iris and a Hamamatsu AC adapter connected to a Power Macintosh 8100/80AV computer. Mean densities of regional 125I-rANP (1–28) binding in the right and left ventricular endocardial lining were analyzed using the PRISM image program (version 3.6–1, Improve Vision, Coventry, UK). Optical densities were measured as disintegrations per minute per square millimeter, based on calibration with the calibration curve derived from the autoradiograms of the 125I standard microscales included in each X-ray cassette. These data were converted into femtomoles of 125I-rANP (1–28) bound per square millimeter, as described elsewhere (3).

The number of ligand binding sites of different affinities, their apparent dissociation constants (Kd), and inhibitory constants (Ki), and their maximal binding capacities (Bmax), which indicate the ability for maximal binding by combination of receptor affinity and population, on particular structures were derived separately in each individual using the LIGAND iterative model-fitting computer program (36).

Determination of particulate GC activity. Endocardial cells were collected from the endocardium of the right ventricle by scraping and isolated by a Percoll gradient centrifugation (purity >90%). The pooled ventricular endocardial cells from three individuals were homogenized at 4°C in 30 mM phosphate buffer (pH 7.2) containing 120 mM sodium chloride and 1 mM phenanthroline by three 30-s bursts of 27,000 rpm using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 1,500 g for 10 min at 4°C, and the supernatant was recentrifuged at 40,000 g for 60 min at 4°C. The membrane pellet was washed three times with 50 mM Tris·HCl (pH 7.4) and resuspended in this solution. Protein contents were determined by a bicinchoninic acid assay kit (Sigma Chemical). Particulate GC activity was
measured according to the method described elsewhere (5, 28). Aliquots of 5 µg of membrane protein were incubated for 15 min at 37°C in a final volume of 125 µl of 50 mM Tris·HCl (pH 7.6) containing (in mM) 1.3-isobutyl-1-methylxanthine, 1 guanosine triphosphate, 0.5 adenosine triphosphate, 15 creatine phosphate, and 4 magnesium chloride with 80 µg/ml creatine phosphokinase, plus ANP(1–28), BNP(1–26), or CNP(1–22) (all 1 µM). Incubations were stopped by adding 375 µl of ice-cold 50 mM sodium acetate (pH 5.8) and boiling for 5 min. Samples were then centrifuged at 10,000 g for 5 min at 4°C.

Production of cGMP was measured in the supernatants by equilibrated RIA. In brief, standards or samples were taken up in a final volume of 100 µl of 50 mM sodium acetate buffer (pH 4.8), and then 100 µl of diluted cGMP antiserum (Calbiochem-Novabiochem, San Diego, CA) and iodinated cGMP (10,000 cpm/100 µl, specific activity 2,200 Ci/mmol, DuPont-NEN, Wilmington, DE) were added and incubated for 24 h at 4°C. The bound form was separated from the free form by charcoal suspension. RIA for cGMP was done on the day of experiments, and all samples in an experiment were analyzed in a single assay. Nonspecific binding was <2.5%. The 50% intercept was at 0.39 ± 0.03 pmol/tube (n = 15). The intra- and interassay coefficients of variation were 6.7 (n = 12) and 8.6 (n = 9), respectively. Average results of determinations were expressed as picomoles of cGMP generated per milligram of protein per minute.

RT-PCR and Southern blot analysis of NPR mRNAs for subtypes. Total RNA was extracted from right ventricular endocardial cells using TRI reagent (MRC, Cincinnati, OH) according to the manufacturer’s protocol. Total RNA concentrations were quantitated by ultraviolet (UV) spectrophotometry. One microgram of total RNA was suspended in ten microliters of buffer containing (in mM) 10 Tris (pH 8.3), 50 KCl, 5 MgCl₂, and 1 each of dATP, dCTP, dGTP, and dTTP. One microgram of total RNA was suspended in ten microliters of buffer containing (in mM) 10 Tris·HCl (pH 8.3), 50 KCl, and 2 MgCl₂ with 200 µM each of dATP, dCTP, dGTP, and dTTP. PCR products were separated in 1.4% agarose gels, and bands were visualized by ethidium bromide staining. Photographs of gels were taken with Polaroid 667 film.

To confirm NPR mRNA, 20 µl of RT-PCR products were subjected to Southern blot analysis. cDNAs of NPRs for subtypes. A hot-start PCR was used to increase the specificity of amplification. The temperature profile of amplification consisted of 30-s denaturation at 95°C, 1-min annealing at 60°C for NPR-A and NPR-B and 65°C for NPR-C, and 2-min extension at 72°C for 40 cycles. PCR products were separated in 1.4% agarose gels, and bands were visualized by ethidium bromide staining. Photographs of gels were taken with Polaroid 667 film.

RESULTS

Localization and specificity of ¹²⁵I-rANP(1–28) binding in ventricles of control rats. Specific ¹²⁵I-rANP(1–28) binding sites were demonstrated in transverse sections of cardiac ventricles using in vitro autoradiography. As shown in Fig. 1A, the comparison of autoradiograms with their corresponding hematoxylin and eosin-stained sections revealed specifically reversible bindings of ¹²⁵I-rANP(1–28) to endocardium of right and left ventricles. No such binding sites were observed in the myocardium of ventricles. In the presence of 1 µM unlabeled rANP(1–28), the dense endocardial binding was completely displaced but the diffuse background binding of radioligand to myocardium was not significantly affected (Fig. 1B). The unrelated peptides, including angiotensin II and arginine vasopressin (both 10 µM), did not inhibit any binding of ¹²⁵I-rANP(1–28) (data not shown). C-ANP, a specific ligand for the NPR-C subtype, competed with the binding of ¹²⁵I-rANP(1–28) to the ventricular endocardium (Fig. 1C).

Comparisons of ¹²⁵I-rANP(1–28) binding characteristics between right and left ventricular endocardium of control rats. In control rats, different binding densities of ¹²⁵I-rANP(1–28) between right and left ventricular endocardium were found (Fig. 1A). As shown in Fig. 2, specific binding to endocardium was much higher in the right than in the left ventricle (P < 0.01): binding densities on the endocardium of the free wall and septal sides were 0.34 ± 0.02 and 0.32 ± 0.02 fmol/mm², respectively, in the right ventricle and 0.16 ± 0.02 and 0.10 ± 0.02 fmol/mm², respectively, in the left ventricle. However, the difference of these binding densities between free wall and septal sides was not significant within each ventricle.

Analysis of the competitive inhibition by unlabeled rANP(1–28) of the binding of ¹²⁵I-rANP(1–28) on right and left ventricular endocardium was consistent with reversible binding sites for rANP(1–28) of uniform affinity on each structure (Fig. 2, insets). The binding affinities of ¹²⁵I-rANP(1–28) were higher in the right than in the left ventricular endocardium: apparent Kd in the free wall and septal sides were 3.07 ± 1.56 and 3.78 ± 1.79 nM, respectively, in the right ventricle and 8.67 ± 2.76 and 40.63 ± 9.65 nM, respectively, in the left ventricle. However, no significant difference of binding capacities of endocardial binding was observed in the right and left ventricular endocardium: Bmax in the free wall and septal sides were 3.76 ± 1.76 and 3.85 ± 1.71 fmol/mm², respectively, in the right ventricle and 4.69 ± 1.96 and 5.05 ± 1.83 fmol/mm²,
respectively, in the left ventricle (Table 1). Therefore, the binding affinities are much higher in the right than in the left ventricle, where $B_{\text{max}}$ are not significantly different from each other.

Increasing concentrations of C-ANP also progressively inhibited the specific bindings of $^{125}\text{I}-\text{rANP}(1–28)$ to these structures (Fig. 3). The binding constants for C-ANP were similar to those for rANP(1–28) (Table 1).

Detection of NPR mRNAs by RT-PCR. The presence of NPR transcripts was tested by RT-PCR. In ventricular endocardial cells, NPR-A cDNA was found in ethidium bromide-stained gels with the expected size of 450 bp (Fig. 4A, lane 2) after 40 cycles of amplification and NPR-B transcripts with 692 bp were also detected (Fig. 4A, lane 4). RT-PCR data from the pituitary gland.

Fig. 1. Dark-field photomicrograph of autoradiograms of cardiac ventricular transverse sections from control rats incubated in presence of 250 pM $^{125}\text{I}$-labeled rat atrial natriuretic peptide (1–28) [rANP(1–28)] (A) and adjacent sections incubated in 250 pM $^{125}\text{I}$-rANP(1–28) + 1 µM unlabeled rANP(1–28) (B) or 10 µM unlabeled des[Gln18,Ser19,Gly20,Leu21,Gly22]ANP(4–23) (C-ANP) (C). Specific $^{125}\text{I}$-rANP(1–28) binding sites appear as white silver grains in right (RV) and left (LV) ventricular endocardium. S, ventricular septum.

Fig. 2. Comparison of competitive inhibition curves of specific $^{125}\text{I}$-rANP(1–28) binding to free wall (A) and septal (B) side of endocardium between RV (●) and LV (▲) of control rats. Mean values from 6 individuals were plotted for competition of binding of 250 pM $^{125}\text{I}$-rANP(1–28) to both sides of RV and LV endocardium by increasing concentrations of unlabeled rANP(1–28). Inset, representative Scatchard plot obtained from 1 of these hearts. B, bound; F, free.
of rats, which expresses NPR-A and NPR-B transcripts (33), were also shown for comparison. NPR-C transcripts of 573 bp were also found (Fig. 4A, lane 6). No transcripts were observed in RNA samples incubated without Moloney leukemia virus reverse transcriptase. The specificity of the PCR products was verified by Southern blotting (Fig. 4B).

Changes of binding densities of 125I-rANP(1–28) in hypertrophied ventricular endocardium of MCT-treated rats. Four weeks after a single subcutaneous injection of 60 mg/kg of MCT, 75% of MCT-treated rats survived and showed liver and lung congestion with pleural effusion. The wall thickness ratio of the right to the left ventricle was 0.62 ± 0.02 (n = 9), approximately twice (P < 0.01 for comparison of right ventricle-to-left ventricle ratios in control vs. MCT-treated rats) that in control rats (0.28 ± 0.02, n = 10). As shown in Fig. 5, the plasma levels of ANP and BNP were about fivefold higher in MCT-treated rats than in control rats.

In MCT-treated rats with right ventricular hypertrophy (Figs. 6 and 7), the specific binding of 125I-rANP(1–28) in both sides of the right ventricular endocardium had actually disappeared (free wall side 0.36 ± 0.02 vs. 0.13 ± 0.04 fmol/mm², septal side 0.35 ± 0.03 vs. 0.12 ± 0.03 fmol/mm²; P < 0.01), but binding in the left ventricle was less affected (free wall side 0.18 ± 0.03 vs. 0.15 ± 0.03 fmol/mm², septal side 0.11 ± 0.02 vs. 0.10 ± 0.02 fmol/mm²).

To determine whether the apparent modulation of specific 125I-rANP(1–28) binding is caused by prior receptor occupancy by endogenous NPs, the heart sections were washed with and without acid solution before incubation with 125I-rANP(1–28). The results of this treatment in control and MCT-treated rats are shown in Fig. 8. The specific binding densities of 125I-rANP(1–28) to ventricular endocardium were markedly increased by acid washing in either control or MCT-treated rats. As shown in Fig. 9A, the increment of specific binding density by acid washing was more remarkable in the left than in the right ventricular endocardium of control rats. However, the increment of specific binding density by acid washing was much higher in the right than in the left ventricular endocardium of MCT-treated rats (Fig. 9B).

Comparison of particulate GC activity between control and MCT-treated rats. The rate of cGMP production

<table>
<thead>
<tr>
<th>Site</th>
<th>rANP(1–28)</th>
<th>C-ANP</th>
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<tr>
<td></td>
<td>Kd, nM</td>
<td>Bmax, fmol/mm²</td>
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<tr>
<td>Right ventricle</td>
<td></td>
<td></td>
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<tr>
<td>Free wall side</td>
<td>3.07 ± 1.56</td>
<td>3.76 ± 1.76</td>
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<tr>
<td>Septal side</td>
<td>3.78 ± 1.79</td>
<td>3.85 ± 1.71</td>
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<tr>
<td>Left ventricle</td>
<td></td>
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<tr>
<td>Free wall side</td>
<td>8.87 ± 2.76</td>
<td>4.69 ± 1.96</td>
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<tr>
<td>Septal side</td>
<td>40.63 ± 9.65</td>
<td>5.05 ± 1.83</td>
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Values are means ± SE; n = 6. Apparent dissociation constants (Kd), inhibitory constants (Ki), and maximum binding capacities (Bmax) were assessed from competitive inhibition of 250 pM 125I-rATP(1–28) binding by various concentrations of unlabeled rANP(1–28) or des[Gln18, Ser19, Gly20, Leu21, Gly22]ANP(4-23) (C-ANP). *P < 0.05 and †P < 0.01 for comparison of corresponding mean values between right and left ventricles.

Fig. 3. Competitive inhibition of maximal specific binding of 250 pM 125I-rANP(1–28) by increasing concentrations of either unlabeled rANP(1–28) (●) or C-ANP (▲). Mean values are shown for free wall (A and C) and septal (B and D) sides of RV (A and B) and LV (C and D) endocardial bindings in control rats (n = 6).
by particulate GC activation of the right ventricular endocardial cell membranes was basally 5.76 ± 1.04 pmol·mg protein⁻¹·min⁻¹. As shown in Fig. 10, CNP(1–22) caused the highest increment of cGMP production, by fourfold over basal levels. In MCT-treated rats with right ventricular hypertrophy, the basal rate of cGMP production was significantly lower than that of control rats (P < 0.05). Even though the cGMP production of the right ventricular endocardial cell membranes was also activated by CNP(1–22), BNP(1–26), and ANP(1–28) at 1 µM concentration, the responses were remarkably suppressed in MCT-treated rats (P < 0.05 vs. control).

**DISCUSSION**

The present study shows the cardiac chamber-specific distribution of NPRs in the ventricular endocardium and their modulation in MCT-treated rats with right ventricular hypertrophy.

 Autoradiograms of the transverse sections of cardiac ventricles revealed that specific ¹²⁵I-rANP(1–28) binding sites were localized in the endocardium of right and left ventricles. This endocardial localization of specific ¹²⁵I-rANP(1–28) binding sites was consistent with previous reports (4, 39). Interestingly, however, our results showed heterogeneity of the binding characteristics of the NPR in the ventricular endocardium. Maximal specific ¹²⁵I-rANP(1–28) binding densities, which indi-
cate the binding of radiolabeled ligand used at present conditions, were much higher in the right than in the left ventricle. Heterogeneity was also observed within the same cardiac chamber. The specific binding densities were significantly lower in the septal side than in the wall side of the left ventricular endocardium, whereas no difference of binding densities was ob-

served between these sides of the right ventricular endocardium. The binding affinities of $^{125}$I-rANP(1–28) were much higher in the right than in the left ventricular endocardium, whereas their maximal binding capacities were not significantly different from each other. Therefore, these results suggest that the heterogeneity of maximal specific $^{125}$I-rANP(1–28) binding densities in ventricular endocardium is related to the properties of binding affinity of the receptor molecules rather than a difference of receptor populations.

Although the presence of mRNA transcripts for NPR subtypes has been noted in the cardiac ventricular tissues (7, 31, 37), the predominance among NPR subtypes in the ventricular endocardium remains to be clarified. Previously, Rutherford et al. (39) found a predominant localization of NPR-C in human ventricular endocardium by quantitative in vitro autoradiography. Consistent with that report, we found that NPR-C is also the predominant NPR subtype in the ventricular endocardium of rats. Specific $^{125}$I-rANP(1–28) binding to ventricular endocardium of the rat heart was displaced progressively by increasing concentrations of C-ANP, a selective ligand for the NPR-C subtype (30, 32, 40). An excess concentration of C-ANP (10 M) inhibited 80–95% of the specific binding of $^{125}$I-rANP(1–28) to ventricular endocardial binding sites; this implies that a very small portion of the specific binding of these structures is NPR-A and/or NPR-B. Therefore, it was technically very difficult to further characterize NPR-A and NPR-B with autoradiographic analysis. We also found mRNA transcripts for three

![Fig. 7. Comparison of specific $^{125}$I-rANP(1–28) binding densities to free wall and septal sides of RV and LV endocardium in control and MCT-induced pulmonary hypertensive rats. Bars are means ± SE of results from acid-washed heart sections (n = 6 for each bar). *P < 0.01 for differences between control and MCT-treated rats.](image)

![Fig. 8. Typical autoradiographic findings for difference of $^{125}$I-rANP(1–28) binding capacities to ventricular sections between values without (A and B) and with (C and D) acid washing in control (A and C) and MCT-induced hypertrophied (B and D) RV endocardium. Acid washing increased $^{125}$I-rANP(1–28) binding densities in the ventricular endocardium of control and MCT-treated rats. S, ventricular septum.](image)
NPR subtypes in ventricular endocardial cells by RT-PCR and Southern blot analysis. Considering that NPR-C has a cellular function as a clearance receptor for endogenous NPs, it is speculated that a predominant localization of NPR-C may protect right ventricular myocardium from high exposure of ANP and BNP originating from the atria.

As mentioned above, it is noteworthy to define the presence of GC-coupled NPRs in the ventricular endocardium. As shown in Fig. 10, equimolar concentrations of ANP, BNP, and CNP increased cGMP production via activation of particulate GC-coupled NPRs of the ventricular endocardial cell membranes, and CNP did this with the greatest potency. The GC-coupled NPRs are subclassified as NPR-A and NPR-B with two monomeric proteins of molecular mass of 120–140 kDa containing an extracellular ligand-binding domain and an intracellular GC catalytic domain (11, 29). The receptor subtypes have been characterized by their ligand selectivity with a rank order of potency for cGMP production; relative potencies for NPR-A and NPR-B are ANP > BNP >> CNP and CNP >> BNP > ANP, respectively (11, 29, 43). Therefore, the present results indicate for the first time that NPR-B is a predominant subtype among the GC-coupled NPRs in the ventricular endocardium. In the cardiac atrium, NPR-B was detected in atrial myocytes by confocal immunofluorescence microscopy (19). Lin et al. (31) showed that cGMP production in ventricular myocytes was stimulated only by ANP and BNP and not at all by CNP. They also presented that cGMP production in ventricular fibroblasts was stimulated by CNP as well as ANP, the latter being only slightly more potent than CNP. In light of these results, it is suggested that the presence of predominant subtype of GC-coupled NPRs in the cardiac ventricle is determined by a mechanism that is cell type specific.

MCT-induced pulmonary hypertension has been a model for experimental right ventricular hypertrophy (10, 27, 35). In the present study, specific $^{125}$I-rANP(1–28) binding to both right and left ventricular endocardium of MCT-treated rats was significantly reduced, with much higher reduction in the right ventricular endocardium. Because the NPR-C may be a major NPR subtype in the ventricular endocardium, the reduction of specific $^{125}$I-rANP(1–28) binding to the endocardium may be related to a change in NPR-C. The modulation in GC-coupled NPRs was also clear in the endocardium of the hypertrophied right ventricle of MCT-treated rats. cGMP production via particulate GC activation by CNP as well as ANP and BNP was markedly decreased. These data are comparable to those of a previous report (7) showing decrease in NPR-C in the hypertrophied ventricular myocardium of rats with aortovenocaval fistula. Our data, however, contrast with the claim in that report of an upregulation of NPR-A and NPR-B of the hypertrophied ventricle. The reason for the discrep-
The ventricular BNP contents were concentration of NPs. Previously, we also found that high levels of plasma NPs and also to the elevated local ventricle of the MCT-treated rat may be related to the specific binding densities in the hypertrophied endogenous NPs. In particular, remarkable decrements of ventricular endocardial cells are indeed exposed to reports (6, 23). This finding suggests that NPRs in ancy may be related to the cell type concerned. Alternatively, the methodology of inducing ventricular hypertrophy may account for the different findings. In the present experiments, acid washing increased specific binding to the NPRs. This is consistent with previous reports (6, 23). This finding suggests that NPRs in ventricular endocardial cells are indeed exposed to endogenous NPs. In particular, remarkable decrements of the specific binding densities in the hypertrophied ventricle of the MCT-treated rat may be related to the high levels of plasma NPs and also to the elevated local concentration of NPs. Previously, we also found that the ventricular BNP contents were ~15-fold higher in MCT-treated rats than in control rats (unpublished data).

In the present experiments, right ventricular hypertrophy was accompanied by an elevation of plasma levels of ANP and BNP. This is consistent with previous reports (2, 16, 24). Increase in plasma levels of ANP may be related to the accentuation of the ventricular and atrial ANP gene expression by cardiac hypertrophy (1, 15, 16, 24, 34). Therefore, it is possible that the downregulation in NPR-A and NPR-B as well as NPR-C may be related to the continuously maintained high levels of plasma ANP and BNP in the ventricular hypertrophy as shown in the present and previous data (2, 16, 24, 34). Furthermore, modulation of NPRs in the ventricular hypertrophy may affect the contractility of the subjacent cardiomyocytes in the heart, because the endocardium is also involved in the regulation of myocardial function as the vascular endothelium regulates performance of subjacent smooth muscle (8).

In conclusion, the present data show heterogeneity in the presence of NPRs in the cardiac chambers, with much higher affinity for NPs in the right ventricular endocardium. The ventricular hypertrophy induced by pulmonary hypertension downregulates the NPRs only in the hypertrophied right ventricular endocardium.

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