Altered expression of natriuretic peptide receptors in proANP gene disrupted mice

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Abstract

Background: The atrial natriuretic peptide (ANP) family is a complex system consisting of at least three polypeptides and at least three types of receptor. Each peptide interacts with different types of receptor at varying degrees of affinity. To determine if natriuretic peptide levels influence natriuretic peptide receptor expression and regulation, we examined the expression of guanylyl cyclase linked GC-A, GC-B and C-receptor in the lungs of mice with a mutation that inactivates the ANP gene (Nppa). Methods: The mRNA level of GC-A, GC-B and C-receptor in the lung were studied by ribonuclease protection assays (RPA). Results: Results of RPA showed that although the mRNA level of GC-A and GC-B of heterozygous ANP 1/2 was not different from wild type ANP 1/1 mice, they were significantly higher in the homozygous mutant ANP 2/2 mice. In addition, C-receptor mRNA level in ANP 1/2 and ANP 2/2 was significantly lower than ANP 1/1 mice. The C-receptor results were confirmed by receptor binding assays and affinity cross-linking studies. Conclusions: Taken together these data suggest that permanent removal of ANP from the natriuretic peptide system results in an up-regulation of GC-A and GC-B, and a corresponding down-regulation of C-receptor in the lung of proANP gene disrupted mice. We postulated that changes in the natriuretic peptide receptor population may result in chronic hypertension and cardiac hypertrophy in the ANP 2/2 mice. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Gene expression; Hypertension; Hypertrophy; Natriuretic peptide; Receptors

1. Introduction

Natriuretic peptides (NPs) are a family of three closely related polypeptides: atrial natriuretic peptide (ANP or A-type), brain natriuretic peptide (BNP or B-type), and C-type natriuretic peptide (CNP). ANP and BNP are cardiac hormones with potent diuretic, natriuretic and vasorelaxant properties that are known to regulate body fluid homeostasis and blood pressure [1] by interacting with at least three distinct natriuretic peptide receptors [2]; whereas CNP is mainly found in the central nervous system [3]. Recent studies indicate CNP may function outside the central nervous system. CNP has been implicated as an endothelial factor that may be involved in the regulation of vascular smooth muscle cell proliferation [4,5]. CNP mRNA has also been localized in the reproductive system of the mouse [6].

The actions of natriuretic peptides are mediated by a family of three receptor types. Two of these receptors (GC-A and GC-B) are guanylyl cyclase-linked and thought to mediate the biological actions of ANP via modulation of cyclic GMP concentrations [7,8]. The GC-A receptor shows high affinity for ANP and BNP, but a 100-fold

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NV and MYT made equal contributions in this investigation.

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lower affinity for CNP. The GC-B receptor exhibits high affinity for CNP and lower affinity for ANP and BNP. The third receptor, C-receptor, which is not linked to guanylyl cyclase, is thought to function as clearance receptor that serves to remove excess natriuretic peptides from the circulation [9]. The C-receptor has similar affinities to all three natriuretic peptides [10].

To help define the exact physiological role of ANP and to understand how natriuretic peptides and their receptors are regulated, a mouse model of ANP gene disruption was established [11]. In the present investigation, we examine the expression of GC-A, GC-B and C-receptor in mice that produce normal level (ANP+/+), half normal level (ANP+/−) or no ANP (ANP−/−) using ribonuclease protection assay (RPA), receptor binding assay, affinity cross-linking study and autoradiography.

2. Methods

Experimental protocols included in this manuscript have been approved by the Animal Care Committee of Queen’s University in accordance with the guidelines of the Canadian Council on Animal Care.

2.1. Peptides and chemicals

Synthetic rANP(99–126) and C-ANP(4–23) (des[Gln18–Ser19–Gly20–Leu21–Gly22]–ANP(4–23)) were synthesized by the Core Facility for Protein/DNA Chemistry, Queen’s University. Chloramine T, phenylmethylsulfonyl fluoride (PMSF), leupeptin, bacitracin, bovine serum albumin (BSA), and sodium lauryl sarcosine were purchased from Sigma Chemicals (St. Louis, MO, USA), disuccinimidyl suberate (DSS) from Pierce Biochemicals (Rockford, IL, USA) and guanidinium thiocyanate, and NaI from ICN (Costa Mesa, CA, USA). RNase T1 and RNase A were obtained from Amersham (Oakville, ON, Canada) and Proteinase K was purchased from Gibco BRL (Burlington, Canada). [α-32P]CTP was purchased from NEN (Guelph, ON, Canada). All other reagents used in this study were reagent grades and purchased from BDH (Mississauga, ON, Canada).

2.2. ProANP gene disrupted mice

The production of the ANP gene disrupted mice has been described previously [11]. In brief, 11 nucleotides of exon 2 of the proANP gene (Nppa) were replaced with the neomycin resistance gene in embryonic stem cells of mouse strain 129. Chimeras harboring the mutation were then mated to mice strain C57BL/6J (B6). Matings between the resulting 129 and C57BL/6J heterozygotes (+/−) produced F2 offspring of all three genotypes (+/+ , +/−, −/−) in Mendelian proportions.

The mice used in this study were obtained from an established colony at Queen’s University by crossbreeding of +/+ mice and the genotype of offspring (i.e. +/+ , +/− and −/−) was determined by Southern blot analysis. F6 to F8 generations of mice of both sexes from 20 to 24 weeks old and weighing 25–35 g were used in this study. The animals were housed according to sex in groups of two to six per cage and kept at an ambient temperature of 22°C and 40% humidity in a room with a 12-h light/dark cycle.

The physical characteristics of the mice at F6 to F8 generations have been published elsewhere [12,13]. Briefly, the arterial blood pressures (in mmHg) obtained from a direct carotid arterial cannula in inactin-anesthetized animals were 95±6 for ANP+/+ and 116±2 for ANP−/− mice (P≤0.0001) and the corresponding heart rate (in beats/min) was 460±30 and 470±20 (P>0.05), respectively. When mice were treated with 8% salt in the diet for at least 2 weeks, the arterial blood pressure (in mmHg) was 113±9 for ANP+/+ and 135±3 for ANP−/− mice.

For genotyping, genomic DNA was isolated from mouse tail by proteinase K digestion followed by a phenol–chloroform extraction. The genomic DNA was digested with EcoRI and electrophoresed on a 0.6% agarose/TBE gel and transferred to a nylon membrane. The membrane was probed with a random-primed, digoxigenin-labeled DNA probe targeted to an 850 bp portion of the ANP gene which extends from exon 1 to exon 3. Specifically-labeled bands were detected by an anti-DIG-AP antibody using enhanced chemiluminescent detection (Boehringer Mannheim, Canada).

Thirty mice were used in this study. Fifteen mice (five from each genotype) were used to determined receptor mRNA levels by RPA, and a similar set of 15 mice were used for in vitro binding and affinity cross-linking assays.

2.3. Studies with ribonuclease protection assay (RPA)

2.3.1. Isolation of cDNA probes for mouse A-, B- and C-type receptor by PCR

The complementary DNA (cDNA) fragments corresponding to the extracellular domain of mouse GC-A, GC-B and C-receptor were obtained from mouse kidney (for GC-B and C-receptor) and atrium (for GC-A) by reverse transcriptase linked polymerase chain reaction (RT-PCR). PCR was carried out using the following synthetic oligonucleotides as primers:

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-A</td>
<td>5′-CCTCCAGTGTTGAAAGTG-3′</td>
<td>5′-GAGGCGACCTGACCGGCT-3′</td>
</tr>
<tr>
<td>GC-B</td>
<td>5′-TTGCCAGAGTGAAGTG-3′</td>
<td>5′-ACGGAACCTGGACCTGGCCG-3′</td>
</tr>
<tr>
<td>C-receptor</td>
<td>5′-CTTCTAGGCCCATGATT-3′</td>
<td>5′-AGAGGCGGTGCGGCTCAA-3′</td>
</tr>
</tbody>
</table>
Fragments of PCR product were then subcloned in pCRII vector (Invitrogen).

2.3.2. DNA sequencing

The identity of the amplified fragments was confirmed by sequencing using M13 forward and M13 reverse primers. DNA sequencing was conducted by the Core Facility for Protein/DNA Chemistry, Queen’s University, Kingston, Ontario, Canada by means of an Applied Biosystems 373A DNA Sequencer.

2.3.3. Tissue and RNA preparation

Tissues were excised from sodium pentobarbital (45 mg/kg) anaesthetized mice, frozen immediately in liquid nitrogen and stored at −80°C until assay. Lungs from individual mice were homogenized with a polytron and the RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method [14] and quantified by spectrophotometry (Hewlett-Packard 8452A).

2.3.4. Ribonuclease protection assay (RPA)

Template cDNA for cRNA synthesis were prepared as follows: (1) in the case of templates for GC-A and GC-B receptor, EcoRI–PstI fragment of exon 1 of GC-A or GC-B were subcloned into plasmid pGEM-3Z (Promega). An AvaI–AvaiI fragment of the C-receptor was subcloned into pGEM-3Z. The GA-C and GC-B plasmids were linearized with Rsal and the C-receptor plasmid was linearized with SacI. Respective antisense cRNA probes were synthesized using [α-32P]CTP as tracer in the presence of T7 RNA polymerase for C-receptor or SP6 RNA polymerase for GC-B and GC-A receptors, and GAPDH (Ambion, Austin, TX, USA). The polyacrylamide gel-purified riboprobes (2.5–5×10^6 cpm) and total RNA (20 μg) were denatured at 85°C for 10 min and annealed overnight at 55°C in 80% formamide, 40 mM PIPES pH 6.5, 0.4 M NaCl, and 1 mM EDTA. Ribonuclease digestion buffer (200 μl; 10 mM Tris pH 7.5, 5 mM EDTA, and 300 mM NaCl) containing 2 μg/ml RNase T1 and 40–50 μg/ml RNase A was then added and incubation continued for 30 min at 30°C. Proteinase K and SDS were added to a final concentration of 0.2 mg/ml and 0.7%, respectively. The mixture was gently mixed and incubated for 30 min at 37°C. After phenol–chloroform extraction, 20 μg tRNA was added and the RNA was precipitated with two volumes of 100% ethanol. The precipitate was washed with 100% ethanol, dried, and resuspended in buffered formamide. Samples were denatured (95°C for 5 min) and electrophoresed on an 8% polyacrylamide sequencing gel. The gels were dried and exposed to Dupont Reflection NEF-469 films with intensifying screen at −70°C.

2.3.5. Analysis of gels

The integrated optical density of the radioactive bands was scanned by a Hewlett-Packard Scanjet IICx/T and analyzed by SigmaGel (Jandel Scientific Software, San Rafael, CA, USA). The reading of each sample was normalized with that of the corresponding GAPDH band.

2.4. Receptor binding studies

2.4.1. Iodination

Iodination of synthetic rANP(99–126) was performed by the chloramine T method as previously described by Sarda et al. [15]. Radiolabeled peptides were purified on a Sep-Pak C18 resin cartridge (Waters Associates, Milford, MA, USA), followed by HPLC on a reversed-phase Vydac C18 column with a linear gradient elution using a solvent system of water/acetonitrile/0.1% trifluoroacetic acid at a flow-rate of 1 ml/min.

2.4.2. Preparation of mouse lung membranes

Frozen mouse lungs were thawed and rinsed in 0.9% saline. The tissue was then minced and homogenized in a Teflon/glass homogenizer with ten volumes of cold 10 mM MgCl2, 10 mM Hepes pH 7.5, 10 mM NaCl, 5 mM PMSF, 5 μg/ml leupeptin and 5 μg/ml pepstatin. The homogenate was centrifuged at 2000×g for 10 min at 4°C and the pellet discarded. The membrane in the supernatant was then pelleted by centrifugation at 16,000×g for 15 min at 4°C. The membrane pellet was collected and then resuspended in 10 mM NaHCO3 at pH 8 containing 5 mM PMSF, 5 μg/ml leupeptin and 0.1% bacitracin and stored at −70°C. Protein concentration was determined by a modified method of Lowry et al. [16] using BSA as a standard.

2.4.3. Receptor binding assays

The method of Weir et al. [10] was used to study receptor binding of ANP in vitro. Briefly, binding assays were performed on ice in a buffer containing 50 mM Hepes pH 7.5, 5 mM MgCl2, 10 mM NaCl, 1 mM PMSF, 2 mg/ml leupeptin, 0.1% BSA and 0.1% bacitracin. The binding reaction was initiated when [125I]rANP(99–126) (60 000–80 000 cpm) and varying amounts of competing, unlabeled ligand were added to 30 μg of lung membranes. Triplicate samples were incubated on ice for 60 min and then centrifuged at 16,000×g for 10 min at 4°C to separate the free from bound peptide. Membrane pellets were washed with 10 mM Hepes containing 10% sucrose at pH 7.5. The resultant membrane pellet with bound, labeled ANP was quantified with a LKB Wallac gamma counter (Model #1275 Minigamma).

2.4.4. Affinity cross-linking studies

Affinity cross-linking studies were performed according to the method of Weir et al. [10]. Briefly, lung membranes pooled from three mice of each genotype (100 μg) were incubated for 1 h on ice in 250 μl of binding buffer (50 mM Hepes pH 7.5, 5 mM MgCl2, 10 mM NaCl, 1 mM PMSF and 0.1% BSA) with 1×10^6 cpm [125I]rANP(99–126)
in the absence or presence of either 1 μM unlabeled ANP or 0.1 μM C-ANP. Samples were centrifuged at 16 000×g for 10 min at 4°C to separate the free from bound peptide. The membrane pellets were washed and resuspended in 50 mM sodium phosphate (pH 7.5). DSS dissolved in DMSO was added at a final concentration of 0.5 mM and samples were incubated at 4°C for 15 min. The reaction was quenched by addition of 1 M Tris (pH 7.0) for 15 min at 4°C and membranes were collected by centrifugation at 16 000×g for 10 min. The membrane pellets were resuspended in SDS gel sample buffer containing 5% (v/v) β-mercaptoethanol and boiled for 5 min. Samples were analyzed by SDS polyacrylamide gel electrophoresis using a 3.5% stack gel and a 7.5% separating gel. Dried gels were exposed to DuPont Reflection NEF-496 film with intensifying screen at −70°C.

2.5. Autoradiographic studies

For autoradiographic studies, mice were prepared according to the method of Pang et al. [17]. Briefly, mice were anaesthetized with sodium pentobarbital (45–50 mg/kg) and the left jugular vein was cannulated for bolus infusion of ANP. Then, 10×10⁶ cpm of [¹²⁵]rANP (99–126) were intravenously injected either alone or with corresponding unlabeled ANP (10 μg) into each mouse. Two min after injection, mice were perfused through the heart first with normal saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Lungs were excised and processed for embedding in paraffin wax. Finally, 4–5-μm sections were cut and mounted onto glass slides, dried, dewaxed, rehydrated in a series of graded ethanol, rinsed in doubled distilled water and air dried. In the dark, the sections were dipped in Amersham LM-1 emulsion and air dried.

After 4 weeks of exposure at 4°C, the slides were developed in Kodak D-19 developer, fixed in sodium thiosulphate and rinsed in distilled water. The slides were then counter-stained with haematoxylin and eosin.

2.6. Statistics

All values were expressed as mean and standard error. Data were analysed by a one-way ANOVA followed by Tukey’s test and a P≤0.05 was considered to be statistically significant.

3. Results

3.1. RPA studies

A comparison of the mRNA levels of the three NP receptors was performed to evaluate the influence of ANP on NP receptor expression. Fig. 1 shows the results of RNase protection assay (RPA) for the mRNA levels of the three NP receptors in the lung for each genotype. The densitometric determination of the relative abundance of GC-A, GC-B and C-receptor mRNA from the RPA experiments are presented in Fig. 2. The results shown in Fig. 2 are normalized for the differences in loading by determining the amount of GAPDH mRNA as an internal control. In homozygous mutant (−/−) mice, expression of the lung C-receptor mRNA was significantly decreased when compared with that of the wild type mice. In the −/− mice, the expression of mRNA for GC-A and GC-B receptors were significantly increased when compared with that in the +/+ mice.
3.2. Binding studies

3.2.1. Competitive binding of ANP to mouse lung membrane

The total binding curves obtained from the equilibrium binding study using rANP \(_{99-126}\) as the unlabeled competing ligand are shown in Fig. 3. The binding affinities for rANP \(_{99-126}\) of each of the three genotypes were very similar, indicating that the natriuretic peptide receptor system remains intact for the ANP gene disrupted mice. IC\(_{50}\) values of 0.32, 0.35 and 0.35 nM were determined for the wild type (+/+), heterozygous (+/−) and homozygous mutant (−/−) mice, respectively.

The total and C-receptor population are shown in Fig. 4. The population of the C-receptor in lung membrane was determined by using C-ANP (a specific ligand for the C-receptor) [9] as the unlabeled competing peptide in the binding study. The total binding sites for the three genotypes of mice were similar. However, a significantly lower binding to the C-receptor was noted in the ANP \(_{2}/_{2}\) as compared with the \(_{1}/_{1}\) and \(_{1}/_{2}\) mice. It is noteworthy that the \(_{1}/_{2}\) mice demonstrated a highest degree of variability for the C-receptor population.

3.2.2. Affinity cross-linking study

Affinity cross-linking of \([^{125}I]rANP_{99-126}\) to the lung membrane indicates the presence of two protein species which bind ANP specifically (Fig. 5, lane 1). The lower (65 kDa) C-receptor band was specifically competed off when C-ANP was used as the unlabeled competing peptide (Fig. 5, lane 2). Competition of both receptors occurred when rANP \(_{99-126}\) was used as the competing ligand (Fig. 5, lane 3). The ratio between GC-A+GC-B and C-receptor of +/+ , +/−, and −/− was 1.1, 2.3 and 3.4, respectively.

3.2.3. Autoradiographic studies

Autoradiographic studies showed that with labeled ANP alone, most of the silver grains were located on the lung parenchyma, intrapulmonary vasculature and very few
were over the bronchial tree (Fig. 6). Virtually no silver grain was detected over the section when both ‘hot’ and ‘cold’ ANP were infused at the same time. There was no detectable difference between the ANP+/+ and ANP−/− mice.

4. Discussion

In order to understand the physiological role of ANP, a mouse model with disruption of the proANP gene has been developed [11]. It has been shown that the homozygous mutant (ANP−/−) mice exhibit elevated arterial blood pressure (compare to the wild type ANP+/+ or heterozygous ANP+/−) and have no circulating or atrial ANP. Furthermore, results from the initial characterization suggest that genetically-reduced production of ANP, as in ANP+/− mice, can lead to salt-sensitive hypertension. A recent report from Melo et al. [12] have confirmed this observation. In addition, they demonstrated that the development of salt-sensitive hypertension in ANP−/− mice is time dependent with a latency of at least 1 week and may be related to their inability to reduce plasma renin activity following high salt treatment.

The present study was designed to determine the expression of natriuretic peptide receptors in the lung of ANP gene disrupted mice. Our hypothesis is that ANP is involved in the regulation of receptor expression under chronic situations. The study by Liu and Yoshimi [19] on DOCA-salt hypertensive rats has shown that the effect of down regulation of the C-receptor in the lung was larger than that in the kidney, suggesting that the lung may play a dominant role in the regulation of the clearance of ANP through C-receptors in vivo. Accordingly, removal of ANP will result in changes in natriuretic peptide receptor expression leading to an altered state of natriuretic peptide function and hypertension. We have chosen the lung as a model system for this study because previous studies have shown a high level of binding of 125I-labeled ANP to this organ and also the lung has been implicated to play a vital role in the clearance of ANP, suggesting that this organ may be important in the overall function of the natriuretic peptide system [17–20].

We have developed a highly sensitive RPA for each of the three natriuretic peptide receptors. Our data demonstrated that the overall animal to animal variability appears to be quite high. The high degree of variability could perhaps be attributed to the differences in the genetic background in the F6 to F8 generation mice used in this study. In spite of the variation in the levels of mRNA within each group, a statistical difference in the levels of mRNA in each genotype does occur. Results show that there is an overall up-regulation of GC-A and GC-B and down-regulation of C-receptor in the ANP−/− mice. Interestingly, GC-A and GC-B levels in ANP+/− were not different from the +/+ mice. On the other hand, the level of C-receptor in the +/− was similar to the −/− mice. It is possible that the reduced C-receptor in the lung of ANP+/− mice is a reflection of the reduced circulating ANP level [11] or modification of expression of BNP and CNP in these animals.

To investigate whether or not down-regulation of C-receptor mRNA is reflected in the overall binding of ANP to its receptors, we studied the binding of labeled ANP with lung plasma membrane preparation using a receptor binding assay. The binding of ANP to C-receptors was determined using C-ANP (a specific ligand for C-receptor) as the unlabeled competing peptide in the assay. Our results show that although the total binding sites for the three genotypes of mice were similar, there was a significantly lower binding of ANP to the C-receptor in the ANP−/− as compared with the +/+ and +/− mice. It therefore follows that there is a corresponding increase in binding of ANP to GC-A and GC-B receptors in the ANP−/−, supporting the findings of the RPA study in the present investigation.
Results from the affinity cross-linking study further substantiate the finding that there is an overall down regulation of C-receptor. When there was no competitor in the assay, the ratio between C-receptor and the GC-coupled receptors in +/+ mice is about 1:1. However, the ratio was much higher (1:3.4) in the −/+ mice, suggesting that the population of the GC-coupled receptors is much higher than that of the C-receptors. In each genotype, ANP binding to the C-receptor was specifically competed off with C-ANP. Interestingly, a faint residual C-receptor band remains in the +/+ mice even after the addition of C-ANP. Subsequent cross-linking studies, where the C-ANP concentration has been increased threefold, has shown a complete competition of the $^{125}\text{Tm}ANP_{99-126}$ binding. Since ANP binds to all NP receptors, all bands disappeared when ANP was used as the competing ligand.

Autoradiographic studies showed that the binding of labeled ANP is mainly in lung parenchyma and intrapulmonary vasculature, but not in the bronchial tree. There were very few silver grains (i.e. binding) present over either the intra-pulmonary bronchi, bronchioles or terminal bronchioles. These data suggest that ANP receptors are mainly located in the pulmonary vasculature and not the conducting portion of the respiratory system in the mouse. Infusion of labeled ANP with excess of unlabeled ANP completely eliminated the binding of ANP in the lung. There is no detectable differences between the binding of ANP in the ANP+/+ and the ANP−/− mice.

Although the relation between the expression of natriuretic peptides and their receptors is not fully understood, studies have suggested that under certain pathological conditions such as hypertension, the elevated level of natriuretic peptides is correlated with a down-regulation of their receptors [21]. Using the ANP gene disruption mice as a model, our data have provided direct evidence that removal of ANP resulted in a down-regulation of C-receptor and a corresponding up-regulation of GC-A and GC-B receptors in ANP(−/−) mice under chronic situations. A down-regulation of the C-receptor would result in a reduced rate of clearance of the remaining natriuretic peptides (BNP or CNP) resulting in an overall amplification of the effect of BNP or CNP.

In summary, we have demonstrated that disruption of the ANP gene results in a redistribution of the three natriuretic peptide receptors in the lung in the absence of ANP. Despite the down-regulation of the C-receptor and a corresponding up-regulation of the GC-coupled receptors, the −/− mice are still chronically hypertensive. The notion whether altered expression of these receptors can lead to chronic hypertension and cardiac hypertrophy in the ANP−/− remains to be determined. Clearly the next logical step is to examine the expression of BNP and CNP in the ANP gene disrupted mouse model to see whether they have been modified in response to the lack of ANP. Overall, the in vivo redistribution of NP receptors in response to the lack of ANP, perhaps, prevents the disruption of the ANP gene from being lethal. Alter-

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Fig. 5. Affinity cross-linking of labeled ANP to isolated lung membranes. Lane 1 represents the in vitro binding of $^{[125]}\text{TmANP}_{99-126}$ in the absence of unlabeled ANP. Lane 2 represents competitive binding of $^{[125]}\text{TmANP}_{99-126}$ in the presence of C-ANP (a ligand which specifically binds only to the C-receptor). Lane 3 represents the nonspecific binding of $^{[125]}\text{TmANP}$ to lung membranes. In this case, 1 μM unlabeled ANP$_{99-126}$ was used to specifically saturate the NP receptors. This figure shows that the ratio between GC-A/GC-B and C-receptor was increased in the +/− and −/− mice, suggesting that the level of C-receptor was reduced in these animals as compared with the +/+ mice.
natively, it is also possible that other endocrine systems such as the renin–angiotensin–aldosterone system may be modified in response to the lack of ANP. Further investigation is required in order to define the relative role of these two systems in blood pressure control and body fluid homeostasis.

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