Renal hyporesponsiveness to atrial natriuretic peptide in congestive heart failure results from reduced atrial natriuretic peptide receptor concentrations

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Bryan PM, Xu X, Dickey DM, Chen Y, Potter LR. Renal hyporesponsiveness to atrial natriuretic peptide in congestive heart failure results from reduced atrial natriuretic peptide receptor concentrations. Am J Physiol Renal Physiol 292: F1636–F1644, 2007. First published January 30, 2007; doi:10.1152/ajprenal.00418.2006.—Atrial natriuretic peptide (ANP) and B-type natriuretic peptide decrease blood pressure and cardiac hypertrophy by activating natriuretic peptide receptor A (NPR-A), a transmembrane guanylyl cyclase also known as guanylyl cyclase A. Inactivation of NPR-A is a potential mechanism for the renal hyporesponsiveness observed in congestive heart failure (CHF) but direct data supporting this hypothesis are lacking. We examined whether NPR-A activity was reduced in CHF, and if so, by what mechanism. In two separate trials, CHF was induced in mice by 8-wk transverse aortic constriction. Sham controls underwent surgery without constriction. The constricted animals developed severe heart failure as indicated by increased heart weight, increased left ventricular end diastolic and systolic diameters, and decreased left ventricular ejection fractions. Kidney membranes were assayed for guanylyl cyclase activity or used to purify NPR-A by sequential immunoprecipitation/SDS-PAGE. Maximal ANP-dependent guanylyl cyclase activities were reduced by 44% or 43% in kidney membranes from CHF animals in two independent trials. Basal cyclase activities were also reduced by 31% in the second trial. The amount of phosphorylated NPR-A was reduced by 25% or 24% in kidney membranes from CHF animals as well. SYPRO Ruby staining suggested that NPR-A protein levels were similar between treatments in the first trial. However, more accurate estimates of NPR-A protein levels by immunoprecipitation/Western analysis in the second trial indicated that NPR-A protein was reduced by 30%. We conclude that reduced NPR-A protein levels, not receptor dephosphorylation, explain the renal hyporesponsiveness to natriuretic peptides in CHF.

hypertrophy; kidney

IN RESPONSE TO ELEVATED BLOOD pressure, atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are released from the atria and ventricles, respectively, into the circulation where they decrease blood pressure and inhibit cardiac hypertrophy (34). The signaling receptor for ANP and BNP is natriuretic peptide receptor-A (NPR-A), which consists of an extracellular ligand-binding domain, a single membrane-spanning region, and intracellular kinase homology-regulatory, hinge-dimerization and guanylyl cyclase-catalytic domains (13, 25, 34). The latter synthesizes the intracellular signaling molecule cGMP, which mediates most natriuretic peptide effects. Previous studies in overexpressing cell culture systems have shown that phosphorylation (37) and dephosphorylation (20, 35) are required for the cellular activation and deactivation of NPR-A, respectively, but receptor phosphorylation state changes have yet to be demonstrated in vivo. Downregulation of NPR-A, as defined by losses of receptor protein in response to ligand binding, is controversial when studied in cell culture. Some groups observe downregulation (31), whereas other groups do not (12, 23). To our knowledge, decreased NPR-A protein levels have not been demonstrated in whole animals. A key target organ of cardiac natriuretic peptides is the kidney. ANP and BNP stimulate natriuresis and diuresis by increasing the glomerular filtration rate and decreasing sodium reabsorption in the collecting duct. ANP also inhibits the secretion of the blood pressure increasing hormone, renin, in the juxtaglomerular cells of the kidney. All of the renal effects appear to be mediated by NPR-A (21).

NPR-A compensates for congestive heart failure (CHF) by reducing preload and afterload as well as inhibiting cardiac hypertrophy (30, 32), consistent with the elevated ANP and BNP levels observed in response to cardiac hypertrophy and/or CHF (15, 39). Despite increased natriuretic peptide levels, the physiological responses to these peptides are blunted in CHF (10, 42). Renal responses such as urine flow rate, urinary sodium excretion, and free water clearance are particularly inhibited in response to infusions of pharmacological doses of ANP in the CHF setting (10, 11, 40). The diminished responsiveness worsens as the patient progresses from compensated to decompensated heart failure despite a continual rise in ANP and BNP levels (7).

Several mechanisms for renal hyporesponsiveness to natriuretic peptides have been suggested including: increased local natriuretic peptide degradation (17, 22), reduced bioactivity of ANP and BNP (16), increased renal degradation of cGMP (28, 44), and downregulation of NPR-A (42, 43). Another mechanism for NPR-A inhibition is receptor dephosphorylation. In response to prolonged ANP exposure or PKC activation, NPR-A dephosphorylation has been correlated with decreased NPR-A guanylyl cyclase activity in cell culture (35, 36).

NPR-A desensitization plays a significant role in the diminished vascular response to ANP because nitric oxide-releasing vasodilators elicit normal vasodilator responses in CHF patients, whereas pharmacological levels of ANP are ineffective (29). In contrast, the contribution of NPR-A desensitization to the diminished renal response is unknown. Here, we show for the first time that renal ANP-dependent cyclase activity is reduced in the CHF setting. To determine whether the reduced activity results from dephosphorylation or downregulation, NPR-A was isolated from kidney homogenates by sequential
immunoprecipitation/SDS-PAGE purification. Receptor protein levels were detected by SYPRO Ruby staining of gels or Western blot analysis. Receptor phosphate levels were determined by ProQ Diamond staining of gels containing fractionated NPR-A immunocomplexes. We find that the diminished ANP-dependent guanylyl cyclase activities in kidneys from animals with CHF result from reduced NPR-A protein not phosphate levels.

MATERIALS AND METHODS

Reagents. Natriuretic peptides and SYPRO Ruby protein gel stain were purchased from Sigma (St. Louis, MO). Radioimmunoassay kit was purchased from Perkin Elmer (Boston, MA). Pro-Q Diamond phosphoprotein gel stain was purchased from Molecular Probes (Eugene, OR).

Aortic banding of mice. Male C57 black mice were anesthetized with a mixture of 80 mg/kg ketamine and 30 mg/kg xylazine intraperitoneally. The neck and upper chest were shaved and a horizontal incision was made at the level of the suprasternal notch to allow direct visualization of the transverse aorta without entering the pleural space. Aortic constriction was performed by ligating the aorta between the right innominate artery and the left carotid arteries over a 26-gauge needle using 5-0 silk suture and a dissecting microscope. The needle was then removed leaving the constriction in place and the skin was closed. For control mice, sham surgeries were performed without constricting the aorta.

Echocardiographic measurement. Echocardiography was performed in mice anesthetized with 1.5% isoflurane by inhalation. Left ventricular (LV) wall thickness, LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD), LV end-systolic wall thickness, and end-diastolic wall thickness were measured using two-dimensional echocardiography. Left ventricular ejection fraction (LVEF) was calculated by the cubic method: LVEF = [(LVEDD)3 - (LVESD)3]/(LVEDD)3 * 100%. LV fractional shortening (FS) was calculated as: FS = (LVEDD - LVESD)/LVEDD * 100%.

Collection of mouse tissues. The mice were anesthetized with a mixture of 80 mg/kg ketamine and 30 mg/kg xylazine intraperitoneally. Kidneys were removed and placed in ice-cold phosphatase inhibitor buffer (4). Whole kidneys were homogenized in 3 ml of phosphatase inhibitor buffer then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was removed and the pellet was washed three times in 2 ml of phosphatase inhibitor buffer by resuspension and centrifugation. After the final wash, the kidneys were resuspended in 1 ml of phosphatase inhibitor buffer and total protein concentrations were determined by the Bradford Method. These membranes were used for cyclase determinations and NPR-A purification without freezing.

Purification of NPR-A. After normalizing for total protein levels, the kidney tissue homogenate was pelleted by centrifugation at 10,000 g for 10 min at 4°C. Each pellet was solubilized in 1 ml of modified RIPA buffer and NPR-A was immunoprecipitated with 3 µl of antiserum from rabbit 6325 in a total volume of 10 ml. Rabbit 6325 was immunized with the synthetic peptide KVRTRYWLLERGCGTRG that corresponds to the last 17 COOH-terminal amino acids of rat NPR-A (2). This antiserum recognizes NPR-A but not NPR-B. The immunoprecipitates were fractionated by SDS-PAGE as previously described (4).

Measurement of guanylyl cyclase activity. Mouse tissue membranes were prepared as described above. Twenty microliters of membranes containing ~80 µg of protein were assayed for guanylyl cyclase activity by addition of GTP/Mg2+ alone (for basal determination) or with GTP/Mg2+ plus 10 nM ANP, 100 nM ANP, or 1 µM ANP. In some assays, activity was assayed in the presence of 1% Triton X-100 and Mn2+ instead of Mg2+ as the cofactor as an indicator of maximal hormone-independent activity. For most assays, the receptor was stimulated by addition of 60 µl of cocktail containing 25 mM HEPES, pH 7.4, 50 mM NaCl, 0.1% BSA, 500 µM IBMX, 1 mM GTP, 5 mM MgCl2, 5 mM creatine phosphate, and 0.1 µg/ul creatine kinase. Membranes were assayed for 1 min and the reactions were stopped with 400 µl of a 50 mM sodium acetate solution containing 5 mM EDTA. One hundred microliters of the reaction were assayed for CGMP concentration in a radioimmunoassay according to the manufacturer’s instructions as previously described (4).

Quantitation of [SYPRO] Ruby, Pro-Q Diamond, and Western blot signal. Gels were stained and amounts of phosphorylated NPR-A and NPR-A protein were quantitated by ProQ Diamond and SYPRO Ruby staining as described (4). Western blotting was performed as described below. Immunocomplexes fractionated by 8% SDS-PAGE were electrotransferred to a PVDF membrane for 20 min at 15 V using a Bio-Rad Trans-Blot SD semidyry transfer cell. The membrane was then blocked in solution of 0.2% casein in PBS for 30 min at room temperature. The blot was then incubated for 2 h in a 1:2,500 dilution of polyclonal rabbit antiserum against NPR-A (antiserum 6325) in TBST at room temperature. The blot was then washed five times in 0.1% Tween in PBS for 4 min each. The blot was then incubated in a 1:20,000 dilution of IRDye 800CW Goat Anti-Rabbit JGg for 45 min at room temperature followed by five washes in PBS containing 0.1% Tween. The blot was imaged on an Odyssey Infrared Imaging System (LI-COR Biosciences).

Statistical analysis. GraphPad Prism software was employed for statistical analysis of the data. Unpaired t-tests were conducted and the mean differences were considered significant when P values were <0.05.

RESULTS

Eight-week transaortic constriction induces cardiac hypertrophy and CHF. Eight weeks after transverse aortic constriction (TAC) surgery, echocardiography was performed on live anesthetized animals to determine LV diastolic and systolic volumes. Mean ejection fraction of the sham-operated animals was 75.6 ± 2.75% compared with 36.5 ± 4.32% in the CHF animals, a reduction of 52% (Fig. 1F). LV fractional shortening was reduced 62% from 37.9 ± 4.66% in the control animals to 14.3 ± 2.07% in the congestive heart-failed mice (Fig. 1F). LV anterior wall thickness increased 22% from 0.068 ± 0.0013 mm in the normal mice to 0.083 ± 0.0060 mm in the congestive heart-failed mice. Posterior LV wall thickness increased 24% from 0.068 ± 0.0013 mm in the control mice to 0.084 ± 0.0060 in the congestive heart-failed mice (Fig. 1E).

When the mice were killed and the hearts were removed, cardiac hypertrophy was clearly evident in the constricted animals (Fig. 1A). The average mass of the hearts from the banded mice (206.1 ± 9.0 mg) was 204% higher than the control mice (101.1 ± 2.0 mg; Fig. 1B). Likewise, the ratio of heart weight to body weight was increased about twofold in the congestive heart-failed mice (Fig. 1C). The wet weight of the lungs from the congestive heart-failed mice (201.3 ± 3.00 mg) was increased 1.5-fold over the control animals (130.6 ± 3.70 mg), consistent with pulmonary edema resulting from CHF. Neither total body weight, kidney weight, nor kidney-to-body weight ratios differed between the two groups (Fig. 1, B and C). Together, these data indicate that the banded animals had reached a state of advanced heart failure.

ANP-dependent guanylyl cyclase activity is reduced in the kidneys of mice with CHF. It has been reported that CHF is associated with loss of responsiveness to ANP and BNP in the kidney (10), but direct measurement of NPR-A guanylyl cy-

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class activities in kidney preparations from congestive heart-failed animals has not been reported. This is critical because it has been suggested that the reduced cGMP levels result from increased natriuretic peptide and cGMP degradation as well as downregulation or dephosphorylation of NPR-A (7). To directly access the effect of CHF on NPR-A, we prepared crude kidney membranes and assayed them for guanylyl cyclase activities in the presence or absence of various activators (Fig. 2). In this assay, basal guanylyl cyclase activities from banded and sham-operated animals were too low to accurately measure. However, we were able to precisely measure cyclase activities in the presence of the nonionic detergent, Triton X-100, and with manganese instead of magnesium as the metal cofactor. These detergent assay conditions are thought to reflect exaggerated basal activities but are more accurate due to the higher levels of cGMP produced under these conditions. Traditionally, we used Triton values to determine whether any changes in natriuretic peptide-dependent activities result from dephosphorylation or losses in receptor protein (35). We found that activities obtained in the presence of detergent and manganese were slightly less in kidneys from the congestive heart-failed animals but the differences were only statistically significant at a P value of 0.21. In contrast, ANP-dependent guanylyl cyclase activities in the kidneys from the congestive heart-failed animals were reduced to 56% of the activity observed in the control kidneys and were statistically significant at a P value of 0.0015.

It has been suggested that the reduced vascular responsiveness to natriuretic peptides results from NPR-A downregulation (ligand-dependent receptor degradation) since 125I-ANP binding to kidney membranes from heart failure animals is decreased compared with binding in membranes from sham animals. However, this conclusion is dubious because the major ANP binding protein in most tissues including kidneys is the nonguanylyl cyclase-linked natriuretic peptide clearance receptor, NPR-C, not NPR-A (27). We reasoned that if NPR-A is internalized and degraded in response to CHF, then the amount of immunoprecipitated NPR-A should be decreased in the banded animals. On the other hand, if receptor dephosphorylation is the mechanism of desensitization, then receptor phosphate levels should be reduced in the absence of any change in receptor protein levels.

To differentiate between these two possibilities, we purified NPR-A from kidneys of congestive heart-failed or sham animals by sequential immunoprecipitation/SDS-PAGE as previously described (4). We then stained the resulting gel with Pro-Q Diamond phosphoprotein dye to assess NPR-A phosphorylation. We rinsed the same gel and stained it with SYPRO Ruby dye to estimate the amount of NPR-A protein present. The negative control for these experiments is immu-
noprecipitation in the presence of preimmune instead of immune serum. The quantitated results of this analysis are shown in Fig. 2, bottom. Total NPR-A protein levels as indicated by SYPRO Ruby staining were unchanged between groups, whereas the phosphate contents as indicated by ProQ Diamond staining were significantly decreased in the kidneys from congestive heart-failed animals. The amount of phosphorylated NPR-A was reduced by 25% in the heart-failed compared with the sham animals. Since the protein levels were unchanged while phosphate levels were reduced, this was consistent with receptor dephosphorylation mediating the reduced ANP-dependent guanylyl cyclase activities. However, since a nonspecific protein comigrated with NPR-A (see preimmune lane of SYPRO Ruby gel), we could not be sure that the SYPRO Ruby data were accurately depicting the amount of NPR-A protein in these membrane samples. Unfortunately, we were unable to confirm these data by immunoprecipitation/Western blot analysis because all of the membranes had been used for the previous assays.

To be sure that the reduced phosphate levels were not a result of reduced protein, we conducted another CHF trial with more animals. However, this time we measured NPR-A protein levels by sequential immunoprecipitation/Western blot analysis using direct infrared fluorescence detection methods. Transaortic constriction was performed on a second group of male C57 black mice and these animals (8 sham, 10 banded) were assessed for signs of heart failure using echocardiography. Left ventricular diastolic and systolic volumes increased significantly in the heart-failed animals (Fig. 3C). The heart weight-to-body weight ratios were also significantly increased 1.75-fold in the congestive heart-failed mice compared with controls, whereas kidney weight-to-body ratios were unaffected (Fig. 3B). The average heart weight (Fig. 3A) of the banded mice (198.0 ± 11 mg) was 167% higher than the control mice (118.7 ± 5 mg). LV anterior wall thickness increased 36% from 0.070 ± 0.0015 mm in the normal mice to 0.095 ± 0.0034 mm in the congestive heart-failed mice. Posterior LV wall thickness was increased 38% from 0.069 ±
0.0011 mm in the control mice to 0.095 ± 0.0035 mm in the heart-failed mice (Fig. 3D). Ejection fractions decreased from 80.0 ± 2.90% in the controls to 40.2 ± 5.25% in the heart-failed animals, a reduction of 50%. Finally, fractional shortening decreased 62% from 43.4 ± 3.10% in the controls to 16.5 ± 2.61% in the congestive heart-failed mice. Based on these parameters, the second group of heart-failed animals showed a similar degree of heart failure as the first group. The only exception being the wet lung weight, which was increased to a statistically significant amount in the congestive heart-failed animals from the first but not the second trial.

**Basal and ANP-dependent guanylyl cyclase activity is reduced in the kidneys of mice with CHF.** After CHF had been confirmed in the second group of banded animals, the mice were killed, their kidneys removed, and guanylyl cyclase assays were performed on membrane preparations from these kidneys. ANP-induced guanylyl cyclase activities were reduced by 27, 33, and 43% at 10, 100, and 1,000 nM concentrations of ANP, respectively, in membranes obtained from congestive heart-failed mice. Based on these parameters, the second group of heart-failed animals showed a similar degree of heart failure as the first group. The only exception being the wet lung weight, which was increased to a statistically significant amount in the congestive heart-failed animals from the first but not the second trial.

**Renal hyporesponsiveness to ANP in CHF is due to decreased NPR-A protein.** In the first trial, we used Pro-Q Diamond stain to determine the phosphorylation state of NPR-A immunoprecipitated from mouse kidneys. Subsequent to the Pro-Q Diamond analysis, we attempted to measure NPR-A protein levels in the kidney by staining the same gel with SYPRO Ruby. We are confident in the use of Pro-Q Diamond as a method for determining phosphate levels as we compared this method directly against the “gold standard,” metabolic labeling of cells with $[^{32}P]$orthophosphate (4). However, in contrast to the clear results obtained measuring endogenous NPR-B protein levels in NIH3T3 cells (38), SYPRO Ruby staining of NPR-A isolated from mice kidneys did not provide a clear, unambiguous signal. Indeed, when preimmune serum was used as a negative control, staining of a nonspecific band was still present in these samples (Fig. 2, bottom). To account for this, the background band was subtracted from the immune samples when the SYPRO Ruby signal was quantitated. The resulting calculations showed no difference in protein levels between the sham and banded animals. However, since the background band was also being subtracted from each sample, we were concerned that real differences between treatments were being masked.

In the second CHF trial, phosphorylated NPR-A was measured by Pro-Q Diamond staining as in the first trial but protein concentrations were determined by immunoblot analysis of NPR-A from a separate gel (Fig. 4, protein signal). The phosphate signal was reduced by 24% in the heart-failed animals compared with the sham mice. However, in contrast to the SYPRO Ruby data from the first trial, the Western blotting data indicated that NPR-A protein levels decrease by 30% in congestive heart-failed mice. The difference was statistically
significant at a *P* value of 0.039. When the average ratios of phosphate to protein were calculated, there was no significant difference between the sham and the heart-failed animals (Fig. 4, middle right). This indicates that the decrease in NPR-A phosphorylation in heart-failed animals is due to decreased protein levels. Hence, reduced NPR-A protein levels, not dephosphorylation, explain the decreased ANP-dependent guanylyl cyclase activities observed in kidneys from mice with CHF.

**DISCUSSION**

In the present study, we report for the first time that renal ANP-dependent guanylyl cyclase activity is reduced in response to transaortic constriction-induced CHF in mice. This is consistent with a previous report showing that urinary cGMP concentrations are decreased in animals with CHF (1). We extended this observation by showing that the reduced cGMP levels result, at least in part, from reduced NPR-A concentrations. This is important because other distinct mechanisms such as increased local natriuretic peptide degradation, increased phosphodiesterase activity, or NPR-A dephosphorylation have been suggested to account for the diminished cGMP response as well (8, 28, 41).

To address the inhibitory mechanism, we measured NPR-A protein and phosphate levels in two separate trials where CHF was induced via transaortic constriction. Two methods, SYPRO Ruby staining and Western blotting, were used to determine NPR-A protein levels in the kidney. Pro-Q Diamond phosphoprotein stain was used in both trials to determine the phosphate content of NPR-A. The phosphorylation of NPR-A decreased by 25 and 24% in trials 1 and 2, respectively; supporting our previous findings that Pro-Q Diamond is a reliable method to determine the phosphorylation status of natriuretic peptide receptors from endogenous sources (4). In the first trial, protein levels measured by SYPRO-Ruby staining showed no change in the receptor levels between the

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**Fig. 4.** Decreased NPR-A protein levels explain the reduced basal and ANP-dependent guanylyl cyclase activities observed in kidney membranes from mice with CHF. **Top:** kidney membranes were prepared and assayed for guanylyl cyclase activities in the presence or absence of various activators. Basal activities were decreased in the banded animals, compared with the sham mice, by 31%. Hormone-dependent activity was reduced by 27, 33, and 43% when stimulated by 10, 100, and 1,000 nM ANP, respectively. *P* value of <0.05 compared with sham. **Bottom:** in CHF, the reduction in the phosphate content of NPR-A is due to decreased receptor levels, as measured by Western blot analysis. NPR-A was immunoprecipitated from the kidneys of sham-operated or banded mice. The immuno-complexes were fractionated by SDS-PAGE on 2 parallel gels. One gel was stained with Pro-Q Diamond phosphoprotein dye to determine phosphate content of NPR-A. The other gel was blotted to a PVDF membrane and probed with a NPR-A-specific antibody followed by a fluorophore-tagged secondary antibody to quantitate protein content. The phosphate signal, protein signal, and the ratio of the phosphate signal to total protein signal were determined and graphed (middle). The amount of phosphate associated with NPR-A was 24% lower in the heart-failed animals compared with the sham-operated animals. In contrast to the previous trial where protein levels were estimated by SYPRO Ruby staining, the more specific immunoblotting method indicated that NPR-A protein levels decreased by 30% in the heart-failed animals. *P* value of <0.05 compared with sham where *n* = 5 for each group.
sham and heart-failed mice. However, the quantitation by this method was encumbered by a nonspecific band that comigrated with NPR-A (see “preimmune” lanes in Fig. 2, bottom), which may explain the lack of any significant differences in NPR-A protein levels between sham and banded animals. Had we based our conclusions on this initial data, we would have erroneously concluded that renal hyporesponsiveness to ANP is due to the dephosphorylation of NPR-A. However, in the second trial, we employed an established method of detecting NPR-A protein levels coupled to a system that produces quantifiable and linear results. It is important to note that in the first trial, preimmune serum was used as a negative control in the immunoprecipitation. In the second trial, a synthetic peptide corresponding to the epitope used to raise the anti-NPR-A serum was preabsorbed to the antibody before the immunoprecipitation. In this lane (see “Peptide Block” lane in Fig. 4, bottom), there is a complete absence of fluorescence signal, and therefore this method of protein quantification is not hindered by nonspecific/background signal as in the first trial with SYPRO Ruby. Using this method, a 30% reduction in NPR-A protein levels was detected in the heart-failed animals compared with the controls. Hence, we conclude that CHF results in reduced hormonal stimulation of NPR-A by a mechanism that primarily involves decreases in receptor protein levels, not receptor phosphate levels. Although NPR-A desensitization has been previously associated with dephosphorylation in cell culture systems (20, 24, 35, 36), dephosphorylation does not appear to account for the losses in NPR-A activity observed in this model of CHF; at least our assays are not able to detect such differences.

Increased levels of circulating ANP and BNP are hallmarks of CHF in the clinic and are routinely used to diagnose heart failure. Despite the increased levels of natriuretic peptides in the failing circulation, the physiological responses to these agents are blunted. Several theories have been proposed to explain this resistance in the kidney (7). One hypothesis is that there is increased local renal ANP degradation by neutral endopeptidases (NEPs). However, neutral endopeptidase inhibitor treatment of severe CHF in dogs was unable to maintain natriuresis (8). Another explanation for the blunted response to ANP and BNP is increased NPR-C expression. However, blocking ANP binding to NPR-C with a specific ligand did not alter renal cGMP production or sodium excretion in dogs with severe CHF (33).

Reduced transcription of NPR-A in response to elevated ANP levels is another explanation for the decrease in ANP-dependent cyclase activity (6). In rat inner medullary collecting duct cells, ANP-dependent NPR-A transcription is decreased in a temporal and dose-dependent manner, leading to decreased de novo receptor synthesis. However, cardiac NPR-A mRNA levels are increased (3, 9) not decreased in response to cardiac hypertrophy, which is not consistent with the reductions in NPR-A protein levels that we observe in the kidney. This could be explained by tissue specificity, but we also observed reduced ANP-dependent guanylyl cyclase activity in heart extracts from congestive heart-failed animals (Dickey DM et al., unpublished observations). Unfortunately, to date, we have not been able to measure receptor protein and phosphate levels in this tissue.

The hypothesis that we currently favor is that NPR-A is inactivated through downregulation, i.e., the receptor is degraded via an endocytotic pathway in a ligand-dependent manner. Since mRNA levels are elevated in tissues where NPR-A activity is decreased, we believe that increased degradation, not reduced synthesis, of NPR-A likely explains the downregulation. Interestingly, most studies in cell culture have indicated that NPR-A is a constitutive membrane resident protein that does not undergo ligand-dependent endocytosis (12, 19, 23, 45). However, the time course of agonist exposure is often short in these assays, which may not represent long-term conditions like CHF. Alternatively, 293 or COS cells may not accurately represent the trafficking properties of NPR-A in vivo. Our current hypothesis is that acute desensitization of NPR-A is mediated by dephosphorylation, whereas activity losses due to chronic conditions result from receptor downregulation. Whether ANP binding and/or cGMP elevations are required for the latter process is currently unknown.

Many studies have shown that the renal response to the cardiac natriuretic peptides, ANP and BNP, are blunted in response to CHF. Whether reduced NPR-A activity contributes to the sodium retention observed in animals or patients with this malady is not completely understood. Although this is an important question, we were unable to add any new information to this problem since we did not measure renal glomerular filtration or sodium secretion rates in our study. However, we were able to add new, definitive information on the role of NPR-A in the diminished response to natriuretic peptides in CHF. Before our study, the contribution of NPR-A to this process had been controversial. In hamsters, investigators found both increased (5) and decreased (26) ANP binding to glomerular membranes from animals with heart failure, whereas in rabbits (18) and rats (14) no effect was observed. Surprisingly, the hormonal responsiveness of the receptor for these peptides has not been directly investigated despite the fact that downregulation is often suggested as a mechanism for the reduced ANP response. In our study, we clearly show that ANP-dependent activation of NPR-A is reduced in the kidneys of animals with CHF. Although we had anticipated that the reductions would be explained by receptor dephosphorylation, our data are not consistent with this hypothesis. In contrast, we found that NPR-A protein concentrations are decreased. This study now sets the stage for future experiments designed to determine the mechanism that mediates the reduced NPR-A levels. Once this process is identified, individual steps in this pathway can be targeted for pharmacologic intervention as a potential treatment for CHF.

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