Decreased Atrial Natriuretic Peptide Binding in Renal Medulla in Rats With Chronic Heart Failure

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The relations between atrial natriuretic peptide (ANP) binding sites in the renal medulla, plasma ANP concentration, and ventricular dysfunction have been studied in rats 4 weeks after myocardial infarction induced by left coronary artery ligation. Plasma ANP concentration was measured by radioimmunoassay, and quantitation of receptors was performed by computerized in vitro autoradiography with 125I-labeled α-rat ANP (1-28) as the radioligand. When compared with controls, rats with myocardial infarction had markedly elevated plasma immunoreactive ANP concentrations (462 ± 82 versus 124 ± pg/ml, p<0.01) and reduced densities of ANP binding in the inner renal medulla (2.93 ± 0.19 versus 3.53 ± 0.22 fmol/mg protein, p<0.01). Extensive myocardial infarction was associated with a significant decrease in receptor numbers in the inner medulla (33.6 ± 5.7 versus 30.5 ± 1.5 X 10^11, p<0.05). Right ventricular weight increased in proportion to infarct size (r=0.71, p<0.01), and both were correlated with plasma immunoreactive ANP levels (r=0.74, p=0.001 and r=0.75, p=0.01, respectively). Binding densities in the inner medulla of rats with infarcts were negatively correlated with right ventricular weight, plasma immunoreactive ANP concentrations, and also with infarct size (r=−0.92, p<0.001; r=−0.78, p<0.001; r=−0.77, p<0.01, respectively). These results suggest that specific binding sites of ANP in the inner medulla decrease in proportion to the elevation in circulating ANP levels, which in turn are related to infarct size and degree of ventricular dysfunction. Decreased ANP binding sites in the kidney may contribute to the blunted natriuretic response to infused ANP in heart failure and may be responsible in part for the impaired sodium and water excretion in chronic heart failure. (Circulation Research 1988;62:155–161)

Atrial natriuretic peptide (ANP) has many physiological actions, which include diuresis, natriuresis, and vasodilation as well as suppression of renin release and aldosterone synthesis. Immunoreactive ANP (IR-ANP) has been demonstrated in both rat and human plasma, and binding sites for rat ANP have been reported in the kidney,6–7 adrenal gland,8,9 and the brain.10 These findings suggest an important role for ANP in the regulation of sodium-water homeostasis and the modulation of blood pressure.

Fluid retention and inability to excrete a sodium load are cardinal features of chronic heart failure. Recent studies have shown that plasma levels of IR-ANP are progressively elevated in proportion to the severity of heart failure in patients11,12 and in rats with chronic heart failure secondary to healed myocardial infarction.13 This may indicate a compensatory effect by ANP to limit salt and water retention in heart failure. However, this compensatory elevation in circulating plasma ANP appears to be inadequate to prevent the abnormal renal handling of sodium and water in chronic heart failure. It has been reported that sensitivity to ANP in the kidney in heart failure is reduced.14–16 One explanation for the reduction in renal sensitivity may be down-regulation of ANP receptors in the kidney. Down-regulation of peptide receptors in response to elevated plasma levels is a well-documented phenomenon noted with other peptide hormones.17

In the present study, quantitative in vitro autoradiography was employed to analyze binding of 125I-labeled α-rat ANP (1-28) in the kidney of a rat model of chronic ischemic heart failure. Furthermore, we have studied the relations between ANP binding in the kidney, plasma IR-ANP levels, and ventricular dysfunction.

Materials and Methods

Induction of Myocardial Infarction

Left ventricular myocardial infarction was produced in female normotensive Wistar rats aged 16–20 weeks by a method previously described.13,18 Briefly, each rat was anesthetized with ether, intubated, and ventilated with a respirator. A left thoracotomy was performed, the heart exteriorized, and the left coronary artery was ligated between the pulmonary artery outflow tract and the left atrium. The heart was then returned to its normal position, the lungs were reinflated with positive end-expiratory pressure, and the thorax was closed by purse-string sutures. The mortality rate for this procedure was 40–60% within the first 24 hours after the
operation. Controls were sham-operated rats in which the operative procedure did not produce a histologically detectable myocardial infarction as a result of failure to occlude the coronary artery.

Four weeks following ligation, the rats were killed by decapitation, and blood was collected into plastic tubes containing disodium ethylenediaminetetraacetic acid (1 mg/ml, EDTA-2Na, AJAX Chemicals Ltd., Sydney), aprotinin (500 kallikrein inhibitor U/ml, Trasylol, Bayer, Leverkusen, Federal Republic of Germany). The heart and kidneys were immediately removed. The left ventricle (including intraventricular septum) was dissected and fixed in 10% buffered formalin for histological study. The infarct size was quantitated histologically by planimetry of serial sections taken from each heart at 1-mm intervals from base to apex by a modified method of Fletcher et al.18 and expressed as a percent of fibrous scar of the endocardial surface.

Measurement of Plasma IR-ANP

Plasma IR-ANP was extracted with Vycor glass beads as reported previously.19 To 1 ml plasma, 30 mg Vycor glass (140 mesh, Society of the Association of Trade with America, Geneva) was added, and absorbed IR-ANP was eluted from the glass powder with 1 ml acetone/water mixture (60:40) containing 0.1% trifluoroacetic acid (TFA, BDH Ltd., Dorset, United Kingdom) and evaporated to dryness and reconstituted in buffer for assay. Recovery of added synthetic α-rat ANP (15–2,000 pg, 28-amino acid, Peptide Institute Co., Osaka, Japan) from rat plasma was linear over this range and averaged 94.0 ± 2.5% (mean ± SEM, n = 8). Serial dilution of the plasma extract showed parallelism to the synthetic ANP standards in the ANP radioimmunoassay.

A 0.1 M Tris-acetate buffer, pH 7.4, containing 0.1% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, Missouri), aprotinin (500 kallikrein inhibitor U/ml), soybean trypsin inhibitor SBTI (50 BAEE U/ml), 0.1% Iso-octylphenoxypolyethoxethanol (Triton X-100, Packard Ins., Downers Grove, Illinois), EDTA-2Na (1 mg/ml), and 0.02% sodium azide, was used to dissolve all reagents and to perform the radioimmunoassay. Fifty microliters of synthetic α-rat ANP or diluted samples were incubated for 48 hours at 4°C with 450 μl buffer, 100 μl antiserum (1:60,000, sheep anti-α-human ANP), and 100 μl 125I-labeled ANP (10,000 counts/min). The separation of antibody-bound and free peptide was performed with goat anti-sheep γ-globulin. The sensitivity of the assay is 6.4 pg α-rat ANP/tube. Intra-assay and inter-assay variances were 8.6% (n = 10) and 14.8% (n = 10), respectively. The anti-human ANP antibody was directed against the COOH-terminal portion of ANP and cross-reacted 100% with α-rat ANP.

Preparation of Radioiodinated α-Rat ANP for Autoradiography

125I-labeled α-rat ANP was prepared by the chloramine T method of iodination, purified by reverse phase high performance liquid chromatography on a Waters C18 μ Bondapak (Millipore, Bedford, Massachusetts) column, and eluted in 27% acetonitrile in 0.05% TFA, to a specific activity of 1,950 Ci/mmol.

Autoradiography of IR-ANP Binding Sites

Autoradiographic techniques were carried out as previously described.15 Kidneys were snap-frozen in isopentane (AJAX) at −40°C, and 20-μm sections were cut in a cryostat (5030 Microtome, Instrument Company Ltd., Huntingdon, United Kingdom) at −20°C, thaw-mounted onto gelatin-coated slides, dried in a dessicator at 4°C for 2 hours, and stored at −70°C until incubation. The sections were preincubated in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 0.2% bovine serum albumin, and 0.05% bacitracin (Sigma) for 15 minutes at 20°C. The sections were then incubated with 125I-labeled α-rat ANP (54 pM) in the same buffer for 1 hour at 20°C. Nonspecific binding was determined in the presence of 1 μM synthetic α-rat ANP. After incubation, the slide-mounted sections were transferred through 4 successive 1-minute washes of 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl at 0°C. The slides were then rapidly dried under a stream of cold air, loaded into x-ray cassettes, and exposed to Agfa Scopix CR3 x-ray film (Agfa Gevaert, Belgium) for 2–6 days in x-ray cassettes at room temperature. A range of known 125I radioactivity standards was applied to 5-mm diameter disks of 20-μm kidney sections and mounted on slides. Each set of radioactivity standards was included in each cassette. These standards were fitted to a calibration curve by a computer to convert optical density values of each pixel into femtomoles of 125I-labeled ANP/mm2.

The x-ray films were processed in a Kodak RP X-omat automatic developer. After exposure, the sections were stained with hematoxylin and eosin. Optical densities were quantitated by computerized densitometry with an EyeCom Model 850 image-analysis system (Log E/ Spatial Data System, Springfield, Virginia) coupled to an 11/23 LSI Computer (Digital Equipment Corp., Marlboro, Massachusetts).

Binding isotherms of renal ANP receptors from 1 control and 1 rat with large myocardial infarction (50% infarct size) were determined with a set of serial sections incubated with 125I-labeled α-ANP and a range of concentrations (10−10 to 10−6 M) of unlabeled ANP. The resulting autoradiographs were quantitated by computerized densitometry, and the data was analyzed by an iterative nonlinear model-fitting computer program.19

Protein content of kidney sections was measured by a modified Lowry method,20 and the area of adjacent serial sections was determined by the image analyzer; the mean value of protein content per unit area was used to convert values of 125I-labeled ANP binding in femtomoles per millimeter squared into femtomoles per milligram of protein.

Statistical Analysis

The data were analyzed with one-way analysis of variance. Duncan's New Multiple-Range test, which
permits multiple testing among treatment means, was then used. Regression lines were fitted by least squares. For statistical analysis of the binding parameters, 95 and 99% confidence limits were established based on log-normal distribution of the parameters. Significance was considered as \( p < 0.05 \). Data are presented as mean ± SEM.

**Results**

**Myocardial Infarct Size and Cardiac Weights**

Healed myocardial infarcts showed a marked thinning of the infarcted area where the muscle was replaced with fibrous connective tissue. Right ventricular weight in rats with infarcts was progressively elevated in proportion to the infarct size \( (r = 0.71, p < 0.01) \) as previously reported. Rats with infarcts were divided arbitrarily into those with less than 30% myocardial infarction \( (n = 7) \) and those with over 30% infarction \( (n = 7) \) as previously described. In these two groups, infarct sizes were 13 ±3% and 45 ±4%, respectively.

Table 1 shows the body and organ weights of control rats and rats with infarcts. There was no significant difference in body weight between controls and rats with infarction. Lung weight was progressively increased in the infarcted groups and correlated significantly with right ventricular weight \( (r = 0.77, p < 0.01) \). Left ventricular weight was also slightly but significantly increased despite marked thinning of the ventricular free wall.

**Plasma IR-ANP**

Mean plasma ANP concentration in the control group was 124 ± 20 pg/ml, similar to that of 10 nonoperated Wistar rats (114 ± 15 pg/ml). Plasma IR-ANP concentrations were significantly higher in the group with large (>30%) infarcts (462 ± 82 pg/ml) than those in the group with small (<30%) infarcts (141 ± 17 pg/ml, \( p < 0.01 \)) and the control group \( (p < 0.01) \). No significant difference was found between the group with small infarcts and the control group (Table 1). Plasma concentrations of IR-ANP were closely correlated with infarct size \( (r = 0.75, p < 0.01) \) and with right ventricular weight \( (r = 0.74, p < 0.01) \).

**Densities of \(^{125}\)I-Labeled ANP Binding in the Renal Inner Medulla**

Labeled ANP binding was observed in both the cortex and the medulla (Figure 2). In the cortex, very high density binding was localized over glomeruli, and a low density of binding was seen in the outer cortex. In the outer medulla, moderate binding occurred over vasa recta bundles, but very high-density binding was observed over the inner medulla. These patterns are identical to those we have previously described and characterized. The inner medulla autoradiographs were relatively uniform in density, so only this anatomical site was subjected to \(^{125}\)I-labeled ANP binding analysis. Nonspecific binding in the presence of 1 μm α-rat ANP was always under 5% of the total binding and did not produce a visible image.

Densities of binding in the inner medulla in the group with large infarcts were significantly reduced compared with those in the group with small infarcts and the control group \( (2.93 ± 0.19 \text{ versus } 3.86 ± 0.14, p < 0.01 \) and versus \( 3.53 ± 0.22 \text{ fmol/mg protein, } p < 0.01 \), respectively; Table 1). There was no significant difference between the group with the small infarcts and the control group. A significant and negative correlation could be demonstrated between plasma IR-ANP concentrations and binding densities in the inner medulla \( (r = −0.78, p < 0.001) \); Figure 3). Densities of binding in the inner medulla were also significantly and negatively correlated with right ventricular weight (Figure 4) and also with infarct size \( (r = −0.92, p < 0.001, r = −0.77, p < 0.01, \text{ respectively}) \).

Analysis of binding isotherms (Figures 5 and 6) showed that a large myocardial infarction induced a significant decrease in binding capacity in the inner medulla compared with a control, 33.6 ± 5.7 (19.7–56.9) versus 95.6 ± 9.6 (69.9–130.4) fmol/mg protein, respectively, \( p < 0.01 \text{ (99% confidence limits)} \), and no significant change was induced in affinity constant, 1.76 ± 0.51 (0.92–3.30) versus 1.03 ± 0.15 (0.74–1.43) \( × 10^4 \text{ M}^{-1} \), respectively, \( p > 0.05 \text{ (95% confidence limits)} \).

To evaluate the possibility that the reduction in the number of binding sites to ligand in the inner medulla...
FIGURE 2. Computer generated pseudo-color images of autoradiographs of $^{125}$I-labeled a-rat ANP (1-28) binding to rat kidney. Red, highest receptor density; blue, low or undetectable receptor density. Top Panel: section from control rat (SHAM). Bottom Panel: section from rat with myocardial infarction (CHF, chronic heart failure). High densities of receptor binding sites are seen in the glomeruli (GL), vasa recta bundles (VRB), and inner medulla (IM). Note the marked difference in the densities in the inner medulla in these sections.

may result from the prior occupancy of receptors in the presence of higher plasma ANP and the lack of dissociation of bound ANP, the following experiment was performed. The kidney sections were incubated in the buffer containing 33 and 267 pM (100 and 800 pg/ml, respectively) synthetic ANP for 30 minutes at 20°C. These concentrations were chosen to mimic plasma levels in control and in rats with the most severe heart failure. They were then preincubated in ANP-free buffer for 15 minutes at 20°C prior to incubation with $^{125}$I-labeled ANP for 1 hour, rinsed at 0°C, removed by wiping with glass microfiber paper (Whatman Ltd., United Kingdom), and counted in a gamma counter. No significant difference was found between 33 (n = 8) and 267 pM (n = 8) ANP-incubated sections. This indicates that the binding results were unlikely to be explained by a possible prior occupancy of the receptor.

Discussion

These results confirm our previous study that demonstrated that infarct size is correlated with the degree of right ventricular hypertrophy and with plasma IR-ANP concentrations.13 This observation supports the current view that plasma ANP concentration increases in proportion to the degree of heart failure, probably via the mechanism of raised atrial pressure and volume.21 Increased circulating ANP levels in chronic heart failure may represent a homeostatic response to increased volume that lessens the salt and water retention of heart failure. However, the presence of prominent edema implies some
Fig. 3. Relation between $^{125}$I-labeled ANP receptor binding densities (fmol/mg protein) in the inner medulla and plasma IR-ANP concentration (pg/ml) ($r = -0.78$, $p<0.001$) in rats with myocardial infarction.

Fig. 4. Relation between $^{125}$I-labeled ANP receptor binding densities (fmol/mg protein) in the inner medulla and right ventricular weight ($r = -0.92$, $p<0.001$) in rats with myocardial infarction.

Fig. 5. Competition curves of the binding of $^{125}$I-labeled ANP to rat inner medulla of a control rat (○) and a rat with myocardial infarction (●). Results are presented as bound-to-total (B/T) ratio versus total ANP concentration (M). Shaded region represents mean ± SEM, 95% confidence limits.

Fig. 6. Scatchard plots derived from $^{125}$I-labeled ANP binding isotherms from sections of renal inner medulla in a control (○) and a rat with myocardial infarction (●). Results are presented as bound-to-free ratio (B/F) versus bound concentration.
physiological state by modulating the actions of ANP at the cellular level.

The finding of specific binding sites of 125I-labeled ANP not only in the renal cortex but also in the renal inner medulla is consistent with previous studies. Binding sites in the cortex are distributed mainly in glomeruli; in contrast, detailed localization of binding sites in the medulla and their physiological role has been uncertain. Recently, autoradiographic techniques combined with microdissection have shown specific binding sites for ANP that overlie the medullary collecting ducts. These observations suggest that the inner medulla may participate in the diuretic-natriuretic action of ANP not only in the renal cortex but also in the renal inner medulla. In addition, Zeidel et al demonstrated the inhibitory action of ANP on sodium entry-dependent oxygen consumption in inner medullary collecting duct cells. In our present data, we have demonstrated a reduction in circulating ANP in plasma may influence the availability of ANP receptors in the kidney in rats with chronic heart failure. This may explain, in part, the blunted response of the kidney to circulating ANP and thus the abnormal sodium handling by the kidney in this pathophysiological condition. The mechanism responsible for these changes remains to be elucidated. Recent observations in an in vitro study by Hirata et al demonstrated that vascular ANP receptors are down-regulated by prolonged exposure to ANP and that receptor-bound ANP is internalized into the cell. These results may provide an insight into the mechanism whereby binding sites of ANP in the kidney are reduced in heart failure.

In conclusion, we have demonstrated a reduction in ANP receptor binding sites in the renal inner medulla in rats with chronic heart failure. This reduction was negatively correlated with plasma IR-ANP levels as well as with the severity of heart failure. Decreased ANP binding sites in the kidney may contribute to the blunted natriuretic response to infused ANP in heart failure and may participate in the impaired sodium and water excretion associated with chronic heart failure.

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References


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**Key Words** • down-regulation • chronic heart failure • salt and water homeostasis • atrial natriuretic peptide • receptor autoradiography
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