Novel Snake Venom Ligand Dendroaspis Natriuretic Peptide Is Selective for Natriuretic Peptide Receptor-A in Human Heart

Downregulation of Natriuretic Peptide Receptor-A in Heart Failure

Gurminder Singh, Rhoda E. Kuc, Janet J. Maguire, Mark Fidock, Anthony P. Davenport

Abstract—The natriuretic peptides are considered to be cardioprotective; however, their receptors have not been identified in human myocardium using radiolabeled analogs. Dendroaspis natriuretic peptide (DNP) has been recently identified as a new member of this peptide family and is thought to be less susceptible to enzymatic degradation. Therefore, we have developed the novel radiolabeled analog [125I]-DNP and used this to localize high-affinity (Kd=0.2 nmol/L), saturable, specific binding sites in adult human heart (n=6) and coronary artery (n=8). In competition binding experiments, atrial natriuretic peptide and brain type natriuretic peptide had greater affinity for [125I]-DNP binding sites than C-type natriuretic peptide and the natriuretic peptide receptor (NPR)-C ligand, cANF. This rank order of potency suggested binding of [125I]-DNP was specific to NPR-A. Messenger RNA encoding NPR-A was identified in left ventricle and coronary artery smooth muscle, and expression was confirmed by immunocytochemical studies at the protein level. In addition, fluorescence dual labeling immunocytochemistry localized NPR-A protein to cardiomyocytes, endocardial endothelial cells, and smooth muscle of intramyocardial vessels. Importantly, we demonstrated a significant downregulation in the density of NPR-A in heart and coronary artery of patients with ischemic heart disease that may explain, in part, the attenuated natriuretic peptide response reported in this patient group. (Circ Res. 2006;99:183-190.)

Key Words: Dendroaspis natriuretic peptide ■ heart failure ■ downregulation ■ natriuretic peptide receptor-A

The heart contributes to the regulation of cardiovascular homeostasis by synthesizing and secreting 2 natriuretic peptide hormones: atrial natriuretic peptide (ANP)1 and brain type natriuretic peptide (BNP).2 ANP and BNP protect the heart from volume overload by inducing potent natriuresis, diuresis, and vasodilatation. Other cardiac protective roles involve attenuation of cardiac hypertrophy, suppression of both the renin–angiotensin–aldosterone system and sympathetic nervous system activity, while enhancing vagal reflexes.3 These effects result in decreased preload and after-load to the heart, which further prevents the development of pathologic cardiac hypertrophy and the progression to heart failure (HF).

In situations of cardiac stress, the heart responds by increasing the secretion of natriuretic peptides from the ventricles, resulting in significantly enhanced circulating levels of ANP and BNP.5 The plasma concentration of these peptides, in particular BNP, has been strongly associated with the degree of left ventricular dysfunction and is being used as a diagnostic and prognostic marker for HF.6 7 Despite the increase in circulating ANP and BNP, the natriuretic, diuretic, and vasodilatory efficacy of these peptides are dramatically reduced in HF.8 9 This may reflect increased metabolism or clearance of natriuretic peptides from circulation and/or downregulation of functional natriuretic peptide receptors (NPRs), although other mechanisms, such as desensitization of NPR-A,10 may also be involved.

Three NPR have been identified to date, 2 of which, NPR-A and NPR-B (also known as GC-A and GC-B, respectively),11 couple to particulate guanylyl cyclase (GC). ANP and BNP bind with high affinity to NPR-A,12 whereas C-type natriuretic peptide (CNP) binds with high affinity to NPR-B.12 The third NPR functions predominantly as a nonspecific natriuretic peptide clearance receptor (NPR-C)13 but has also been shown to activate the pertussis toxin-sensitive protein G,14 Although NPR-C is nonselective for the endogenous natriuretic peptides, the ANP analog des-[Glu18Ser19Gly20Leu21Gly22]-ANF-(4-23)-NH2 (cANF) demonstrates higher selectivity for NPR-C over the other NPR.12

In adult human heart, binding sites for [125I]-ANP have been detected on the endocardial endothelial cells15 but not to the myocardium. This is surprising because mRNA transcripts for all 3 NPRs have been identified in the myocardium,16 consistent with in vitro studies17,18 that demonstrate that...
ANP potently attenuates hypertrophy of human cardiomyocytes. Therefore, despite considerable research into the cardiovascular physiology of natriuretic peptides, determination of NPR distribution, density, and identity at the protein level in adult human heart and also in the vasculature has been inconclusive.15,19,20 Hence, there is little information regarding the characterization of NPR-A in adult human heart and whether progression to HF alters NPR-A density, which may contribute to the reduced efficacy of endogenous and exogenous natriuretic peptides in patients, where synthetic BNP (nesiritide) has been approved for the treatment of acute decompensated HF (http://www.fda.gov/cder/approval/index.htm).

Recently, a novel natriuretic peptide was identified from the venom of the Green Mamba snake (Dendroaspis angusticeps) and consequently named Dendroaspis natriuretic peptide (DNP).21 Although a DNP homolog has not yet been identified by bioinformatic analysis of the human genome, immunoreactivity (IR) to this peptide has been detected in human tissues,22 whereas circulating levels have been demonstrated to correspond to the degree of HF.23 The physiological function of DNP is similar to ANP, inducing natriuresis24 and vasodilatation.22 Interestingly, neutral endopeptidase 24.11(NEP) does not affect DNP-mediated effects in animals,25 suggesting that DNP may have enhanced resistance to degradation. We hypothesized, therefore, that a radiolabeled analog of DNP could be used to localize the distribution of NPR-A and to determine whether receptor density is altered in HF.

Therefore, we synthesized a novel radioiodinated analog of DNP, [125I]-DNP (Figure 1), and our aims were to (1) determine whether [125I]-DNP can be used to visualize receptors in adult human heart tissue; (2) characterize receptors in adult human left ventricle and coronary artery based on pharmacological criteria of saturable, specific and high-affinity binding characteristics; (3) determine the identity of [125I]-DNP binding sites; (4) confirm the distribution of NPR-A in human heart and coronary artery using molecular biological and immunocytochemical techniques; and (5) monitor alterations of receptor density in left ventricle and coronary artery of patients with HF.

### Materials and Methods

An expanded Materials and Methods section is in the online data supplement, available at http://circres.ahajournals.org

#### Tissue Samples

Human heart tissue and coronary artery were obtained from patients, with informed consent and local ethical approval, undergoing cardiac transplantation for either dilated cardiomyopathy (DCM) (n=12) or ischemic heart disease (IHD) (n=14) or were from donor heart (n=10), for which there were no suitable recipients. The coronary artery from DCM patients was considered to be histologically normal.26 All tissues were sectioned at 30-μm thickness. Patient details are summarized in Table 1.

#### Cardiovascular Tissue Profiling by Receptor Autoradiography

[125I]-DNP (2000 Ci/mmol) (Amersham Biosciences, GE Healthcare, Bucks, UK) was prepared from unlabeled DNP by direct iodination with sodium [125]-iodide using the chloramine-T method. Sections of left ventricle, right ventricle, left atria, right atria, and coronary artery from donors and intraventricular septum (containing the conducting system) from DCM patients were preincubated with 50 mmol/L Tris-HCl buffer, containing 10 mmol/L MgCl2 and 5 mmol/L ethylenediaminetetraacetic acid (pH 7.2) (binding buffer). Sections were then incubated with binding buffer containing 0.2 nmol/L [125I]-DNP, for 1 hour, at 22°C. Nonspecific binding was determined by inclusion of 10 μmol/L DNP. After washing, sections were air-dried and apposed to radiation sensitive film for 3 days.

#### [125I]-DNP Saturation Binding Studies

Left ventricle and coronary artery from donors and patients with DCM or IHD were sectioned. Following a 15 minute preincubation with binding buffer, sections were incubated with increasing concentrations (2 pmol/L to 1 nmol/L) of [125I]-DNP, for 1 hour, at 22°C. Nonspecific binding was determined by inclusion of 10 μmol/L DNP. After washing, sections were air-dried and apposed to radiation sensitive film for 3 days.

#### Table 1. Summary of Patient Characteristics

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Age (y)</th>
<th>Sex (Male/Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>10</td>
<td>40±5</td>
<td>5/5</td>
</tr>
<tr>
<td>DCM</td>
<td>12</td>
<td>50±2</td>
<td>9/3</td>
</tr>
<tr>
<td>IHD</td>
<td>14</td>
<td>52±1</td>
<td>12/2</td>
</tr>
</tbody>
</table>

IHD and DCM patients were on a combination of angiotensin-converting enzyme inhibitors, aldosterone inhibitors, Ang II receptor antagonists, antirhythmic, anticoagulants, β-blockers, Ca2+ channel blockers, diuretics, lipid-lowering agents, nitrates, and nonsteroidal antiinflammatory drugs.
washing, sections were measured for radioactivity using a gamma counter.

Specificity of [125I]-DNP Binding
Sections of left ventricle were preincubated with binding buffer and then incubated with 0.2 nmol/L [125I]-DNP in the presence and absence of 1 μmol/L of the structurally related peptides ANP, BNP, CNP, and DNP as well as structurally unrelated peptides angiotensin II (Ang II), apelin-13, bradykinin (BK), endothelin-1 (ET-1), ghrelin, guanylin, somatostatin-14 (SST-14), and vasoactive intestinal peptide (VIP), at 22°C, for 1 hour. After washing, sections were measured for radioactivity using a gamma counter.

Competition Binding Studies
Following preincubation with binding buffer, sections of left ventricle were incubated with 0.2 nmol/L [125I]-DNP in the presence of increasing concentrations of the NPR-A agonists, ANP and BNP (2 pmol/L to 3 μmol/L), the NPR-B agonist, CNP (2 pmol/L to 10 μmol/L), the NPR-C ligand cANF (2 pmol/L to 10 μmol/L), or DNP (2 pmol/L to 3 μmol/L), for 1 hour, at 22°C. After washing, sections were measured for radioactivity using a gamma counter.

Immunocytochemistry
Acetone fixed sections of left ventricle were blocked for nonspecific protein interactions with 5% swine serum and incubated with rabbit anti–NPR-A (human) serum for 72 hours (4°C) at 1:300 dilution in PBS solution/Tween (PBS/T) containing 2% swine serum. After washing in PBS/T, sections were incubated for 1 hour, at 22°C, with 1:200 dilution of swine anti-rabbit serum. Sections were washed and incubated with 1:400 dilution of rabbit peroxidase/antiperoxidase complex for 1 hour at 22°C. Following a final wash, NPR-A–like IR was visualized by incubating sections with 3,3′-diaminobenzidine in 0.05 mol/L Tris-HCl buffer containing 0.3% H2O2.

Fluorescence Dual Staining and Confocal Microscopy Image Analysis
Cryostat-cut sections of left ventricle and coronary artery were fixed in acetone, blocked with 5% goat serum, and incubated with rabbit anti–NPR-A serum at 1:300 dilution and either mouse anti–von Willebrand factor (vWF) monoclonal antibody at 1:50 dilution or mouse anti–smooth muscle α-actin (SMAa) at 1:100 dilution, in PBS/T containing 1% goat serum, for 72 hours, at 4°C. After washing in PBS/T, sections were incubated with the secondary antibody solution containing both AlexaFluor 488 conjugated goat anti-rabbit serum (1:200 dilution) and AlexaFluor 568 conjugated goat anti-mouse serum (1:200 dilution), for 1 hour, at 22°C. Following a final wash, sections were mounted using Vectashield mounting medium and images analyzed.

RT-PCR Assays
Left ventricle, right atria, and coronary artery smooth muscle mRNA was extracted using Invitrogen Micro-FastTrack 2.0 Kit according to the protocol of the manufacturer. Synthesis of cDNA was performed using Invitrogen SuperScript III First-Strand Synthesis System. RT-PCR conditions and specific primers for human NPR-A were designed based on published data.27 Control reactions were performed with primers for β-actin on cDNA. PCR products were separated by electrophoresis on a 1% agarose gel with ethidium bromide. RT-PCR primer–specific reaction product sizes were estimated by reference to a 100-bp DNA molecular weight standard.

Data and Statistical Analysis
The data from binding studies were analyzed using the iterative, nonlinear curve fitting programs in the KELL package (Biosoft, Cambridge, UK). Pooled Kd, Bmax (normalized to fmol/mg protein), and Hill slope were expressed as mean±SEM. Data were compared using Student’s 2-tailed unpaired t test as appropriate. Multiple comparisons were evaluated by ANOVA. Statistical significance was taken as P<0.05.

Results
Cardiovascular Tissue Profiling by Receptor Autoradiography
Specific [125I]-DNP binding was observed throughout the myocardium of the left ventricle, right ventricle, left atria, right atria, and intraventricular septum (Figure 2A through 2E). Within the left ventricle, [125I]-DNP binding was also identified to small intramyocardial vessels and to endocardial endothelial cells (Figure 2F). In histologically normal epicardial coronary artery, specific [125I]-DNP binding was detected to the vascular smooth muscle (Figure 2G).

[125I]-DNP Saturation in Adult Human Histologically Normal Left Ventricle and Coronary Artery
To ascertain the binding characteristics of [125I]-DNP, saturation experiments were performed on normal left ventricle and histologically normal epicardial coronary artery. Over the concentration range tested, [125I]-DNP bound monophasically to both tissues with saturable and comparable subnanomolar affinity (Table 2). Hill coefficients were close to unity and confirmed the radioligand binds with a single affinity.

Specificity of [125I]-DNP Binding
At fixed concentration (1 μmol/L) of ANP, BNP and CNP competed significantly for 0.2 nmol/L [125I]-DNP binding.
(P<0.05), to a comparable extent as 1 μmol/L unlabeled DNP (Figure 3). Binding of CNP was significantly less (1-way ANOVA: P<0.005) when compared with the other natriuretic peptides. The structurally unrelated peptides did not compete for [125I]-DNP binding, suggesting binding of [125I]-DNP was specifically to NPR.

[125I]-DNP Competition Binding in Human Heart

To verify the identity of [125I]-DNP binding sites, competition binding experiments were performed. The NPR-A agonists ANP and BNP competed for [125I]-DNP binding in a monophasic fashion and with comparable nanomolar potencies of 26.8±4.3 and 21.5±1.8 nmol/L (Figure 4A). Unlabeled DNP competed with similar characteristics to ANP and BNP, with a potency of 12.1±3.2 nmol/L. CNP and cANF also competed monophasically for [125I]-DNP binding but in comparison with unlabeled DNP, had ~70- and 30-fold lower potency of 825.4±174.9 and 367.9±77.5 nmol/L, respectively (Figure 4B). The rank order of potency was DNP>BNP≈ANP>cANF>CNP, which indicates that [125I]-DNP was binding to NPR-A.

RT-PCR Assay

RT-PCR primer–specific reaction products of the expected size for NPR-A were detected in human left ventricle, right atria, and smooth muscle of coronary artery (Figure 4C).

Primer-specific reaction products for β-actin, used as a positive control, were detected in cDNA. Bands were not visible when primers for NPR-A and β-actin were omitted.

**Immunocytochemistry**

NPR-A IR was localized to cardiomyocytes and small intramyocardial vessels of left ventricle (Figure 5A and 5B) received from donor hearts. No staining was detected in adjacent sections when the primary antibody was omitted (Figure 5C).

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**Figure 3.** Specificity of 0.2 nmol/L [125I]-DNP in sections of adult human left ventricle (n=4). ANP, BNP, CNP, and DNP (all 1 μmol/L) competed significantly for [125I]-DNP binding sites, but the structurally unrelated peptides, including VIP, which is a ligand for NPR-C, did not compete. [125I]-DNP binding is specific to NPR-A or NPR-B. *P<0.05 vs [125I]-DNP, †P<0.005 CNP vs ANP, BNP, DNP (1-way ANOVA).

**Figure 4.** [125I]-DNP competition binding study. ANP (n=4), BNP (n=4), and DNP (n=3) compete with similar high affinity for [125I]-DNP binding sites (A), whereas CNP (n=4) and cANF (n=4) compete with lower affinity (B). The derived rank order of affinity was DNP>BNP≈ANP>cANF>CNP. C, RT-PCR primer–specific reaction products (arrow, 491 bp) for human NPR-A detected from RNA extracted from coronary artery (CA) smooth muscle (n=3), left ventricle (LV) (n=3), and right atria (RA) (n=3). Primers for β-actin were used as positive control (+) on cDNA. Bands were not visible when primers were omitted (-).
Fluorescence Dual Staining and Confocal Microscopy Image Analysis

The distribution of NPR-A was further determined using fluorescence dual-labeling techniques, revealing that IR to NPR-A colocalized with SMαA IR in intramyocardial vessels (Figure 5D through 5F). However, NPR-A IR did not colocalize with vWF IR in the endothelium of these vessels (Figure 5G through 5I). In contrast, NPR-A IR did colocalize with vWF IR in the endocardial endothelial cells of the left ventricle (Figure 5J through 5L). Furthermore, the green fluorescence signal for NPR-A IR displayed striation-type patterns in the tissue surrounding intramyocardial vessels, which is typical of a cardiomyocyte distribution. In epicardial coronary artery, NPR-A IR did not colocalize with vWF IR in the endothelium (Figure 6D through 6F) but did colocalize with SMαA IR (Figure 6A through 6C).

Figure 5. A and B, Representative photomicrographs showing NPR-A IR in adult human left ventricle to cardiomyocytes (cm) (n=4) and intramyocardial vessels (imv) (n=4). C, NPR-A IR was absent when the primary antibody was omitted. D through L, Photomicrographs showing confocal images of fluorescent dual-labeling immunocytochemistry in adult human left ventricle. NPR-A IR, shown in green (D), colocalized with SMαA IR, shown in red (E), to intramyocardial vessels (n=4) (F). NPR-A IR (G) did not colocalize with vWF IR, shown in red (H), to endothelium of intramyocardial vessels (n=4) (I). NPR-A IR (J) colocalized with vWF IR (K) to the endocardial endothelial cells (n=2) (L). Scale bar=200 μm (A through C); scale bar=25 μm (D through L).

Figure 6. Photomicrographs (A through F) showing confocal images of fluorescent dual-labeling immunocytochemistry in adult human epicardial coronary artery. NPR-A IR (A) colocalized with SMαA IR (B) in epicardial coronary artery (C). NPR-A IR (D) did not colocalize with vWF IR (E) in these vessels (F). Scale bar=25 μm (A through F).
Downregulation of \([^{125}I]\)-DNP Binding Density in Heart Failure

We measured the density of NPR-A in the coronary artery and left ventricle of HF patients to determine whether alterations in NPR-A density may contribute to the significant attenuation of natriuretic peptide efficacy reported in this patient group. \([^{125}I]\)-DNP binding was saturable and bound with high affinity to left ventricle from DCM and IHD patients (Table 2). The affinity of binding in both patient groups was comparable to that in donor left ventricle. However, the density of binding sites \((B_{\text{max}})\) was significantly \((P<0.005)\) downregulated in left ventricle of patients with IHD.

Compared with histologically normal epicardial coronary artery, the density of binding sites was significantly \((P<0.05)\) downregulated in coronary artery from patients with IHD, without significant alterations in the affinity of binding (Table 2). Hill coefficients were close to unity in all saturation binding experiments, suggesting binding of \([^{125}I]\)-DNP was with a single affinity.

Discussion

We have reported for the first time the binding characteristics of the novel snake venom ligand \([^{125}I]\)-DNP in adult human left ventricle and coronary artery. \([^{125}I]\)-DNP binding to left ventricle and coronary artery was saturable, specific, and occurred with high affinity. Additionally, binding of \([^{125}I]\)-DNP was detected throughout human heart myocardium, endocardial cells, and small intramyocardial vessels. In epicardial coronary artery, binding of \([^{125}I]\)-DNP to the smooth muscle was detected. We have established the identity of these \([^{125}I]\)-DNP binding sites as NPR-A using specificity and competition binding experiments. Furthermore, we confirmed the presence of RT-PCR primer–specific reaction products for NPR-A in human ventricular and atrial tissue and in the smooth muscle of coronary artery. Using immunocytochemistry, we determined the cellular distribution of NPR-A and detected NPR-A IR on cardiomyocytes of the left ventricle, small intramyocardial vessel smooth muscle, epicardial coronary artery smooth muscle, and endocardial endothelial cells, consistent with our distribution of \([^{125}I]\)-DNP binding sites. Importantly, we have demonstrated a significant downregulation in the density of NPR-A in left ventricle and coronary artery from patients with IHD.

The initial aim of our study was to ascertain whether \([^{125}I]\)-DNP could be used to determine the distribution of NPR in adult human heart tissue. Profiling of cardiovascular tissue, using receptor autoradiography, demonstrated that \([^{125}I]\)-DNP binding sites were present throughout the myocardium of human heart. Binding of \([^{125}I]\)-DNP to small intramyocardial vessels, to smooth muscle of large epicardial coronary arteries, and to endocardial endothelial cells was also detected. This latter observation was in agreement to a previous study using \([^{125}I]\)-ANP, where binding sites were only detected in adult human endocardial endothelial cells.\(^{15}\) Therefore, our study overlaps with and greatly expands on the distribution of NPR in adult human heart tissue.

The density of NPR-A, measured by \([^{125}I]\)-DNP in the left ventricle \((\approx 6 \text{ fmol/mg protein})\), was comparable to that of the Ang II receptors \((\approx 3 \text{ fmol/mg protein})\)\(^{28}\) and also to receptors for the emerging cardiovascular peptides apelin \((\approx 8 \text{ fmol/mg protein})\)\(^{29}\) and ghrelin \((\approx 6 \text{ fmol/mg protein})\).\(^{30}\) In contrast, the density of NPR-A in both the left ventricle and coronary artery \((\approx 8 \text{ fmol/mg protein})\) was \(\approx 10\)-fold lower than the density of ET-1 receptors in these tissues \((\approx 65 \text{ fmol/mg protein})\)\(^{11}\) and \(\approx 71 \text{ fmol/mg protein}\)\(^{32}\) in human left ventricle and coronary artery, respectively.

In specificity experiments, only the 3 endogenous natriuretic peptides, ANP, BNP, and CNP, inhibited \([^{125}I]\)-DNP, whereas a range of structurally unrelated cardiovascular peptides did not compete. Interestingly, ANP and BNP inhibited binding of \([^{125}I]\)-DNP comparable to DNP, suggesting that NEP does not differentially influence the binding of these peptides to receptors on left ventricle. VIP, a ligand with high affinity for NPR-C,\(^{14}\) was inactive, confirming that \([^{125}I]\)-DNP radioligand was not binding to NPR-C. Importantly, guanylin, which binds another member of the particulate GC-receptor family, GC-C,\(^{33}\) did not compete. At the highest concentration tested \((1 \mu\text{mol/L})\), all 3 natriuretic peptide competed for \([^{125}I]\)-DNP binding, with CNP inhibiting binding to a lesser extent, consistent with rank orders of potency reported for these peptide at the NPR-A receptor.\(^{34,35}\)

In left ventricle, ANP, BNP, and DNP competed monophonically with comparable affinity for \([^{125}I]\)-DNP binding sites. In contrast, CNP competed with \(\approx 30\)-, \(40\)-, and \(70\)-fold lower affinity when compared with ANP, BNP, and DNP, respectively. Similarly, cANF competed with \(\approx 14\)-, \(17\)-, and \(30\)-fold lower affinity in comparison with ANP, BNP, and DNP, respectively. The rank order of potency from our study was DNP > BNP > ANP > cANF > CNP, which is consistent with the rank order of potency of agonists that would be expected for NPR-A.\(^{12}\) Our data therefore provided strong evidence for the specific binding of \([^{125}I]\)-DNP to NPR-A in human heart. This is also in agreement with the reported action of DNP in inducing natriuresis\(^{34,35}\) and vasodilatation,\(^{32}\) both considered to be attributable to NPR-A activation.

The expression of NPR-A in adult human heart was confirmed using immunocytochemistry, which paralleled the distribution visualized by \([^{125}I]\)-DNP, with NPR-A IR present throughout the ventricular myocardium. The identity of cells expressing the receptor was determined and included the endocardial endothelial cells and smooth muscle cells of intramyocardial vessels, but not the endothelium, the latter in agreement with functional studies.\(^{36}\) Comparable distribution of NPR-A IR was visualized in epicardial coronary artery, colocalizing with the smooth muscle but not to the endothelium of these vessels. To confirm that these tissues were capable of synthesizing the receptor, mRNA encoding NPR-A was detected in the smooth muscle of coronary artery, left ventricle, and right atrium, consistent with the literature.\(^{46,37}\) The presence of NPR-A receptors in myocytes as well as endocardial and smooth muscle cells suggests that the latter cell types may potentially also contribute to the significant downregulation in receptor number in the myocardium of patients with IHD.

The physiology of NPR-A agonists, such as ANP, in the vasculature is well established, inducing potent and efficacious endothelium independent vasodilatation, in both small resistance vessels\(^{36}\) and large conduit arteries.\(^{36,38}\) Further-
more, it has recently been reported that endogenous ANP modulates the smooth muscle tone of smaller veins and venules.\(^9\) This suggests that NPR-A may be important in the regulation of vascular smooth muscle tone in a range of vessels. Interestingly, in patients with HF, the efficacy of both endogenous and exogenously infused natriuretic peptide induced vasodilatation is significantly reduced\(^{9,40}\) when compared with healthy volunteers. In our study, we report that the density of NPR-A in coronary artery is significantly downregulated in IHD patients compared with histologically normal coronary artery. Therefore, this may result in loss of natriuretic peptide induced vasodilatation in coronary arteries supplying the heart.

In contrast to the vasculature, the role of natriuretic peptides on ventricular hemodynamics is less well understood. Although ANP is regarded as a negative inotrope, data supporting this is inconsistent and often contradictory.\(^41\) However, in vitro studies\(^{17,18}\) have demonstrated natriuretic peptides to have potent antihypertrophic effects on cardiomyocytes. Mouse knockout models of the gene encoding NPR-A (\(Npr1\)) validate these in vitro studies, as \(Npr1^{-/-}\) mice develop hypertension, cardiac fibrosis, and exaggerated cardiac hypertrophy.\(^{42,43}\) To verify that hypertrophy is not the consequence of hypertension, a recent study developed a mouse model with selective disruption of \(Npr1\) in cardiomyocytes.\(^{44}\) These mice did not develop hypertension but exhibited significant cardiac hypertrophy. In agreement with these in vivo and in vitro studies, the size of cardiomyocytes was significantly reduced in mice with cardiomyocyte-specific overexpression of \(Npr1\) when compared with wild-type mice.\(^{45}\)

Mechanisms other than reduction of NPR-A density may also contribute to the reduced efficacy of natriuretic peptides in HF patients. Both NPR-C density and mRNA have been reported to be significantly increased in platelets\(^{46}\) and left ventricle,\(^{47}\) respectively, of patients with HF. The latter study also monitored mRNA expression levels of NPR-A in left ventricles of nonfailing and IHD patients but found no significant differences. This suggests that the alterations in receptor density observed in the present study involve post-translational mechanisms. Furthermore, NEP activity is also significantly increased during progression to HF.\(^{48}\) Therefore, our observed downregulation in NPR-A density in patients with IHD, together with receptor desensitization\(^{10}\) as well as increased metabolism and clearance of natriuretic peptides following release from the cardiomyocytes, may all contribute to the reported blunted natriuretic peptide response in patients with HF.

In conclusion, we have synthesized and characterized a novel radioligand, \([^{25}\text{I}]\)-DNP, and quantified, for the first time to our knowledge, NPR-A in adult human heart and cardiac vessels. Cells expressing NPR-A were identified by immunocytochemistry. Using this radioligand, we have discovered the density of NPR-A was significantly reduced in cardiac tissues from patients with IHD. \([^{25}\text{I}]\)-DNP will allow further quantification of changes in NPR-A to understand the role of these receptors in both human disease and in animal models following targeted disruption of the natriuretic system.

Acknowledgments
We thank Emma Mead for providing RT-PCR data and Jean Chadderton and the consultant and theater staff of Papworth Hospital for tissue collection.

Sources of Funding
This work was supported by the British Heart Foundation. G.S. was supported by a Biotechnology and Biological Sciences Research Council Cooperative Awards in Science and Engineering studentship with Pfizer.

Disclosures
None.

References


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Circ Res. 2006;99:183-190; originally published online June 15, 2006; doi: 10.1161/01.RES.0000232322.06633.d3

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Data Supplement (unedited) at:
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Expanded Materials & Methods

Cardiovascular Tissue Profiling by Receptor Autoradiography

Cyrostat cut sections (30 µm) of fresh-frozen human left ventricle, right ventricle, left atria, right atria and coronary artery from donors and intraventricular septum (containing the conducting system) from DCM patients were thaw mount onto gelatin coated microscope slides. These were allowed to air dry briefly and stored at -70°C until required. Frozen sections were thawed at room temperature and pre-incubated with 50 mmol/L Tris-HCl buffer, containing 10 mmol/L MgCl₂ and 5 mmol/L ethylenediaminetetracetic acid, (pH 7.2) (binding buffer), for 15 min at 22°C, to remove endogenous natriuretic peptides and degradative enzymes. Pre-incubation buffer was removed and sections were incubated with binding buffer containing 0.2 nmol/L [125I]-DNP, for 1 hour, at 22°C. In adjacent tissue sections, non-specific binding was defined at each concentration of [125I]-DNP by inclusion of 1 µmol/L DNP. Equilibrium was broken by washing in 50 mmol/L Tris-HCl buffer (pH 7.4) at 4°C for 3 x 5 minutes and rinsing in deionised water to remove buffer salts. Sections were dried rapidly in a stream of cold air prior to apposing to radiation sensitive film for 3 days. Autoradiographic films were developed manually (Kodak D-19, Unifix).

[125I]-DNP Saturation Binding Studies

Cyrostat cut sections (30 µm) of fresh-frozen human left ventricle and coronary artery from donors and patients with DCM or IHD were thaw mounted onto gelatin coated microscope slides. These were allowed to air dry briefly and stored at -70°C until required. A further three sections per tissue were collected into microcentrifuge tubes to measure protein (in order to determine B_max expressed as fmol/mg of protein). Frozen sections were thawed at room temperature and pre-incubated with 50 mmol/L Tris-HCl buffer, containing 10 mmol/L MgCl₂ and 5 mmol/L ethylenediaminetetracetic acid, (pH 7.2) (binding buffer), for 15 min at 22°C, to remove endogenous natriuretic peptides and degradative enzymes. Pre-incubation buffer was removed and sections incubated with binding buffer containing increasing concentrations (2 pmol/L – 1 nmol/L) of [125I]-DNP, for 1 hour, at 22°C. In adjacent tissue sections, non-specific binding was defined at each concentration of [125I]-DNP by inclusion of 1 µmol/L DNP. Equilibrium was broken by transferring slides to racks and washing in 50 mmol/L Tris-HCl buffer (pH 7.4) at 4°C for 3 x 5 minutes. Each section was wiped from the slide with a filter paper circle and transferred to a counting tube and counted in a gamma counter to measure DPM.
Data were analyzed using the non-linear iterative curve fitting programs in the KELL package (Biosoft, http://www.Biosoft.com) containing the EBDA (Equilibrium Binding Data Analysis) and LIGAND programs and normalized to protein content in fmol/mg protein. EBDA performed the preliminary analysis of saturation data, converting radioactivity in DPM into molar concentrations of ligand. Hill slopes were calculated separately and a Scatchard transformation was used to provide initial estimates of $K_D$ and $B_{max}$. LIGAND used files created by EBDA, together with initial estimates of the binding parameters, to fit the data to a one or multiple binding site model. A weighted, non-linear curve fitting routine was iteratively refined to provide more accurate estimates of the $K_D$ and $B_{max}$ values than those obtained with linear (Scatchard) transformations alone. LIGAND calculated an F value, taking into account the improvement in the goodness of fit that accompanied an increase in the number of parameters to be fitted in the two-site model compared with a one-site fit. The program indicated which of the two models is statistically the better fit.1

**Specificity of $[^{125}\text{I}]-\text{DNP}$ Binding**

Cryostat cut sections (30 µm) of fresh-frozen human left ventricle were thaw mount onto gelatin coated microscope slides. These were allowed to air dry briefly and stored at -70°C until required. Frozen sections were thawed at room temperature and pre-incubated with binding buffer for 15 minutes to remove endogenous natriuretic peptides and degradative enzymes. Sections were then incubated with 0.2 nmol/L $[^{125}\text{I}]-\text{DNP}$ in the presence and absence of 1 µmol/L of the structurally related peptides ANP, BNP, CNP and DNP as well as structurally unrelated peptides angiotensin-II (Ang-II), apelin-13, bradykinin (BK), endothelin-1 (ET-1), ghrelin, guanylin, somatostatin-14 (SST-14) and vasoactive intestinal peptide (VIP), at 22°C, for 1 hour. Equilibrium was broken by washing in 50 mmol/L Tris-HCl buffer (pH 7.4) at 4°C for 3 x 5 minutes. Each section wiped from the slide with a filter paper circle and transferred to a counting tube and counted in a gamma counter to measure DPM. Data were expressed as % total $[^{125}\text{I}]-\text{DNP}$ binding.

**Competition Binding Studies**

Cryostat cut sections (30 µm) of fresh-frozen human left ventricle were thaw mount onto gelatin coated microscope slides. These were allowed to air dry briefly and stored at -70°C until required. A further three sections per tissue were collected into microcentrifuge tubes to measure protein. Frozen sections were thawed at room temperature and pre-incubated with binding buffer for 15 minutes to remove endogenous natriuretic peptides and degradative enzymes. Sections were then incubated with 0.2 nmol/L $[^{125}\text{I}]-\text{DNP}$ in the presence of
increasing concentrations of the NPR-A agonists, ANP and BNP (2 pmol/L – 3 µmol/L), the NPR-B agonist, CNP (2 pmol/L – 10 µmol/L), the NPR-C ligand cANF (2 pmol/L – 10 µmol/L), or DNP (2 pmol/L – 3 µmol/L), for 1 hour, at 22°C. Non-specific binding was determined by inclusion of 1 µmol/L cold DNP in adjacent sections. Equilibrium was broken by washing in 50 mmol/L Tris-HCl buffer (pH 7.4) at 4°C for 3 x 5 minutes. Each section was wiped from the slide with a filter paper circle and transferred to a counting tube and counted in a gamma counter to measure DPM. Data were analyzed using the non-linear iterative curve fitting programmes in the KELL package.¹

Immunocytochemistry

Cryostat cut sections of human left ventricle were thaw mounted onto poly-L-lysine coated microscope slides and stored at -70°C until required. Prior to use, sections were transferred to slide racks and air-dried for 18 hours. Sections were then fixed in acetone for 10 minutes (4°C). Non-specific staining was blocked by incubating sections with 5% swine serum in phosphate buffered saline solution (PBS). Blocking reagent was gently tipped off and sections were then incubated with the primary antibody, rabbit anti-NPR-A (human) serum (raised against human sequence of NPR-A294-308: LKQLKHLAYEQFNFT) for 72 hours (4°C) at 1:300 dilution in PBS/Tween (PBS/T) containing 2% swine serum. For negative controls, adjacent tissue sections were incubated in PBS/T in 2% swine serum in the absence of the primary antibody. After washing in PBS/T (3 x 5 minutes, 4°C), sections were incubated for 1 hour, at 22°C, with 1:200 dilution of swine anti-rabbit serum containing 2% swine serum. Sections were washed again in PBS/T (3 x 5 minutes, 4°C) and then incubated with 1:400 dilution of rabbit peroxidase/antiperoxidase complex in PBS/T containing 2% swine serum for 1 hour at 22°C. Following a final wash in PBS/T (3 x 5 minutes, 4°C), NPR-A like immunoreactivity (IR) was visualized by incubating sections with 3,3'-diaminobenzidine in 0.05 mol/L Tris-HCl buffer containing 0.3% H₂O₂. The reaction was stopped by gently flooding the sections with de-ionized water. Sections were dehydrated along an alcohol gradient and cleared in xylene for 1 hour. The sections were then mounted using DePeX-Gurr mounting medium and examined using a standard bright field microscope.²

Fluorescence Dual Staining and Confocal Microscopy Image Analysis

Cryostat cut sections of human left ventricle and coronary artery were thaw mounted onto poly-L-lysine coated microscope slides and stored at -70°C until required. Prior to use, sections were transferred to slide racks and air-dried for 18 hours. Sections of left ventricle and coronary artery were fixed in acetone (4°C) for 10 minutes
and then incubated with 5% goat serum in PBS to block non-specific protein interactions. Blocking reagent was gently tipped off and sections were incubated with rabbit anti-NPR-A serum at 1:300 dilution and either mouse anti-von Willebrand Factor (vWF) monoclonal antibody at 1:50 dilution or mouse anti-smooth muscle α-actin (SMαA) at 1:100 dilution, in PBS/T containing 1% goat serum, for 72 hours, at 4°C. After washing in PBS/T (3 x 5 minutes, 4°C), sections were incubated with the secondary antibody solution containing both AlexaFluor 488 conjugated goat anti-rabbit serum (1:200 dilution) and AlexaFluor 568 conjugated goat anti-mouse serum (1:200 dilution), for 1 hour, at 22°C in PBS/T containing 1% goat serum. Following a final wash (3 x 5 minutes, 4°C), sections were mounted using Vectashield mounting medium and confocal imaging was performed using a confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).2

Reverse Transcriptase-Polymerase Chain Reaction Assays

Left ventricle, right atria and coronary artery smooth muscle mRNA was extracted using Invitrogen Micro-FastTrack 2.0 Kit according to manufacturer’s protocol. Synthesis of cDNA was carried out using Invitrogen SuperScript III First-Strand Synthesis System. Reverse transcriptase-polymerase chain reaction (RT-PCR) conditions and specific primers for human NPR-A were designed based on published data3 (forward primer 5’>CTTGGGGAGAGGGGGAGTAGCAC<3’, reverse primer 5’>GGGGGTCGGGGGAGCAGGTATTGT<3’). The PCR cycle consisted of a denaturation stage at 94°C for 1 minute, an annealing stage at 62.5°C for 1 minute and a polymerization stage at 72°C for 1 minute. This was repeated for 30 cycles with the final polymerization stage extended for an addition 5 minutes.3 Control reactions were performed with primers for β-actin on cDNA. PCR products were separated by electrophoresis on a 1% agarose gel with ethidium bromide. RT-PCR primer specific reaction product sizes were estimated by reference to a 100bp DNA molecular weight standard.

Materials

[125I]-DNP (2000 Ci/mmol) (Amersham Biosciences, GE Healthcare, Bucks, U.K.) was prepared from unlabelled DNP by direct iodination with sodium [125I]-iodide using the chloramine-T method. The peptides Ang-II, ANP, BK, CNP, ET-1, ghrelin, SST-14 and VIP were from the Peptide Institute (Osaka, Japan). Apelin-13, CNP, cANF and guanylin were from BACHEM (Merseyside, U.K.). DNP was from Phoenix Pharmaceuticals (Belmont, CA, U.S.A). Rabbit anti-NPR-A serum was from Abcam (Cambridge, U.K.). The secondary antibodies rabbit-PAP and swine anti-rabbit serum were from DAKO (Ely, U.K.). AlexaFluor 488
conjugated goat anti-rabbit serum and AlexaFluor 568 conjugated goat anti-mouse serum were obtained from Molecular Probes (Leiden, The Netherlands). Invitrogen Micro-FastTrack 2.0 Kit, Invitrogen SuperScript III First-Strand Synthesis System and NPR-A primer set were from Invitrogen (Paisley, U.K.) All other reagents were from Sigma-Aldrich (Poole, U.K.).

**Data and Statistical Analysis**

The data from binding studies were analyzed using the iterative, non-linear curve fitting programs EBDA and LIGAND in the KELL package (Biosoft, Cambridge, UK). Individual saturation binding experiments were analyzed with EBDA to obtain initial estimates of binding parameters. These EBDA files were then co-analyzed with LIGAND to obtain values of ligand affinity (K_D) and receptor density (B_max) expressed as fmol/mg of protein). A two-site model was only accepted if it resulted in a significantly better fit determined using the F-test. Pooled K_D, B_max and Hill slope were expressed as mean±SEM. Data were compared using Student’s 2-tailed unpaired t-test as appropriate. Multiple comparisons were evaluated by ANOVA. Statistical significance was taken as P < 0.05.

**References**

