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# Guanylyl cyclase (GC)-A and GC-B activities in ventricles and cardiomyocytes from failed and non-failed human hearts: GC-A is inactive in the failed cardiomyocyte

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## Abstract

Cardiomyocytes release atrial natriuretic peptide (ANP) and B-type natriuretic peptide to stimulate processes that compensate for the failing heart by activating guanylyl cyclase (GC)-A. C-type natriuretic peptide is also elevated in the failing heart and inhibits cardiac remodeling by activating the homologous receptor, GC-B. We previously reported that GC-A is the most active membrane GC in normal mouse ventricles while GC-B is the most active membrane GC in failing ventricles due to increased GC-B and decreased GC-A activities. Here, we examined ANP and CNP-specific GC activity in membranes obtained from non-failing and failing human left ventricles and in membranes from matched cardiomyocyte-enriched pellet preparations. Similar to our findings in the murine study, we found that CNP-dependent GC activity was about half of the ANP-dependent GC activity in the non-failing ventricular and was increased in the failing ventricle. ANP and CNP increased GC activity 9- and 5-fold in non-failing ventricles, respectively. In contrast to the mouse study, in failing human ventricles, ANP-dependent activity was unchanged compared to non-failing values whereas CNP-dependent activity increased 35% (p=0.005). Compared with ventricular membranes, basal GC activity was reduced an order of magnitude in membranes derived from myocyte-enriched pellets from non-failing ventricles. ANP increased GC activity 2.4-fold but CNP only increased GC activity 1.3-fold. In contrast, neither ANP nor CNP increased GC activity in equivalent preparations from failing ventricles. We conclude that: 1) GC-B activity is increased in non-myocytes from failing human ventricles, possibly as a result of increased fibrosis, 2) human ventricular cardiomyocytes express low levels of GC-A and much lower levels or possibly no GC-B, and 3) GC-A in cardiomyocytes from failing human hearts is refractory to ANP stimulation.



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**Keywords**

Natriuretic peptide receptor A; Carperitide; Nesiritide; Natriuretic peptide; Heart failure; cGMP

## 1. Introduction

Three genetically distinct, but structurally similar, natriuretic peptides called atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) are expressed in humans [1]. ANP and BNP activate guanylyl cyclase (GC)-A, whereas CNP activates the homologous receptor, GC-B [2,3]. Synthetic versions of ANP called carperitide and BNP called nesiritide are approved for the treatment of acutely decompensated congestive heart failure in Japan and the USA, respectively [4].

ANP and BNP are released from the heart in response to volume and pressure overload, which increase cardiac transmural distending pressure. Circulating peptide levels are highly correlated with the severity of systolic heart failure and serum BNP concentrations are routinely used to diagnose heart failure [5]. In the early stages of heart failure, the natriuretic peptide system provides a myriad of compensatory actions, which are dependent upon ANP and BNP activating GC-A in target tissues. These compensatory actions include vasodilation, opposition of the renin–angiotensin–aldosterone system, attenuated sympathetic nervous system activation, and renal actions that promote natriuresis.

In chronic heart failure, the effects of ANP and BNP are attenuated despite high circulating natriuretic peptide concentrations assessed by commercial assays [6–9]. Several possible explanations exist for the blunted natriuretic peptide response including: increased local natriuretic peptide degradation [10,11], reduced bioactivity of ANP and BNP [12], increased release of less active forms of BNP [13–15], increased degradation of cGMP [16,17] and reduced GC-A activity due to receptor dephosphorylation and/or degradation [6,18,19]. However, very little information is available regarding activity changes in GC-A and GC-B that result as a function of human heart failure.

CNP is released from endothelial cells [20] and its importance to the compensatory actions of the natriuretic peptide system in heart failure is incompletely understood. CNP levels are elevated in patients with advanced systolic heart failure and are positively correlated with mean pulmonary capillary wedge pressure and the severity of heart failure [21,22]. CNP is expressed in rodent cardiomyocytes [23] and signals in an autocine/paracrine manner within the myocardium to oppose the development of cardiac hypertrophy and fibrosis in response to pressure-overload. For example, rats expressing a transgenic dominant negative form of GC-B displayed reduced CNP-dependent guanylyl cyclase activity, progressive blood pressure-independent cardiac hypertrophy and increased heart rate [24]. The transgenic animals also displayed marked hypertrophy in response to pressure overload, consistent with previous data showing that CNP inhibits cardiomyocyte hypertrophy in culture [25].

To examine natriuretic peptide receptor activity in heart failure, we previously measured ANP-GC-A-dependent and CNP-GC-B-dependent guanylyl cyclase activities in ventricular membranes from control and heart-failed mice [26]. We found that CNP-dependent activity

was about 70% of the ANP-dependent activity in the normal heart and that ANP-dependent activity was markedly reduced while CNP-dependent activity was slightly elevated in the failing ventricle. In the current study, we examined the more important question of what happens to natriuretic peptide-dependent GC activity in response to human heart failure. These studies used both membranes prepared from human left ventricles and membranes derived from cardiomyocyte enriched cell pellets isolated from the same hearts. Similar to the murine study, we found that CNP-dependent GC activity represented a substantial portion of the ventricular GC activity and was increased in the failed ventricle. We also found that GC-A activity was present at lower levels in cardiomyocyte-enriched pellet preparations while GC-B activity was just detectable. Finally, GC activity in myocytes from failing hearts was unresponsive to natriuretic peptide stimulation.

## 2. Materials and methods

### 2.1. Reagents

Human natriuretic peptides (ANP and CNP) were purchased from Sigma-Aldrich (St. Louis, MO). The cGMP radioimmunoassay kits were purchased from Perkin Elmer (Boston, MA).

### 2.2. Collection of tissues

Human tissue samples were obtained from patients with end-stage heart failure undergoing heart transplantation and non-failing donors deemed unsuitable for transplant. Prospective informed consent for use of heart tissues for research was obtained from all transplant recipients or from the next-of-kin in the case of organ donors. The Institutional Review Board at the University of Pennsylvania and the Gift of Life Donor Program (Philadelphia, PA) approved the use of human heart tissue for this research. All hearts were arrested in situ using a cold, high potassium, cardioplegia solution. Hearts were kept on wet ice until further processing (<3 h), as previously described [27]. One section of transmural left ventricular tissue was flash-frozen as a whole tissue ventricular sample while an adjacent left ventricular free wall region was used for cell isolation.

Cardiomyocytes were isolated using a 3-step perfusion digestion protocol as previously described [27]. Briefly, a catheter was placed in an epicardial branch artery for the perfusions. Digestions included a 30 min perfusion with crystalloid buffer to clear the retained blood cells, a 30 min collagenase perfusion, and a final rinse phase. After passage through a stainless steel mesh (200 μm), the crude tissue digestion mixture from the mid myocardium of the left ventricular free wall was collected, diluted in 150 to 250 ml resuspension buffer and subjected to gravity sedimentation. Cadiomyocyte-enriched pellets were obtained after 10 min of sedimentation.

### 2.3. Preparation of membranes

Frozen ventricular or cardiomyocyte-enriched pellet samples were thawed on ice and then homogenized in phosphatase inhibitor buffer as previously described [28]. The samples were then centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatant was removed and the pellet was washed 3 times in phosphatase inhibitor buffer by resuspension and centrifugation. After the final wash the samples were resuspended in phosphatase inhibitor

buffer. The protein concentrations of the membranes were adjusted to 5.5–7.5 mg/ml for the ventricular samples and 3.2–5.6 mg/ml for the cardiomyocyte-enriched pellets and then used for guanylyl cyclase determinations without freezing.

### 2.4. Measurement of guanylyl cyclase activity

Membrane fractions containing approximately 125 μg [range=110 to 150] of protein for the ventricle samples and 90 μg [range=70 to 111] of protein for the cardiomyocyte samples were assayed for guanylyl cyclase activity by addition of $Mg^{2+}$GTP alone (for basal determinations) or with $Mg^{2+}$GTP and 1 μM ANP or 1 μM CNP. The receptor was stimulated by addition of 60 μl of a reaction mixture containing 25 mM HEPES, pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM IBMX, 1 mM ATP, 1 mM GTP, 5 mM $MgCl_2$, 5 mM creatine phosphate and 0.1 mg/ml creatine kinase. The reaction was conducted for 5 min at 37 °C. In some assays, $Mg^{2+}Cl_2$ and ATP were removed and 1% Triton X-100 and 3 mM $Mn^{2+}$GTP was substituted. All reactions were stopped with 0.4 ml of ice-cold 50 mM sodium acetate solution containing 5 mM EDTA. One-fifth of the reaction was removed and assayed for cGMP concentrations by radioimmunoassay as previously described [28].

### 2.5. Statistical analysis

Statistics and graphs were generated with Prism 5 software. P-values were obtained using Student's unpaired t-test where $p \leq 0.05$ was considered significant. The vertical bars within the symbols represent the SEM.

## 3. Results

### 3.1. Characteristics of tissue donors

Ventricular samples were obtained from patients with and without heart failure and frozen in liquid nitrogen as described under Materials and methods. The characteristics of the donating individuals are shown in Table 1. All failing hearts came from individuals with severe symptoms of heart failure with all being inotrope-dependent. The average left ventricular ejection fraction in the failing group was 23±4%. Of the 7 heart failure patients, 3 had ischemic heart failure and 4 had non-ischemic heart failure. The non-failing hearts were obtained from organ donors with brain death due to trauma or cerebrovascular accidents with no clinical history of heart failure. Echocardiograms were available for 7 of 10 non-failing subjects and revealed an average left ventricular ejection fraction of 52±9%.

### 3.2. Human ventricular membranes contain both ANP-GC-A and CNP-GC-B guanylyl cyclase activity

Crude membranes were prepared from the frozen ventricular samples by thawing in a buffer optimized for maximum guanylyl cyclase activity. We previously demonstrated that this buffer, which contains serine–threonine phosphatase inhibitors and low magnesium concentrations, produced more highly phosphorylated and more active receptors than a commonly used buffer that lacks phosphatase inhibitors and EDTA [29]. Maintaining GC-A and GC-B in a highly phosphorylated state is essential to measure maximum guanylyl cyclase activity because the hormone-dependent activity of these receptors is directly and positively correlated with the phosphate content of the receptors [30,31].

GC activities were measured under basal conditions (with $Mg^{2+}$ GTP and 1 mM ATP) or under the same conditions with the addition of saturating concentrations (1 μM) of human ANP or CNP. As a control, GC activity was also determined in the presence of 1% Triton X-100 and 3 mM $Mn^{2+}$GTP in the absence of $Mg^{2+}$. These conditions activate all GCs to near maximal levels [32]. These "Triton values" are best thought of as elevated basal estimates and are an excellent indicator of the total amount of GC activity in any given preparation due to the accuracy associated with the higher GC measurements. GC activities assayed in the presence of Triton X-100 and $Mn^{2+}$ were not significantly different between non-failing and failing ventricular samples, which indicated that the protein assays on the individual samples were uniform and accurate.

Basal GC activity measured in the human ventricular membrane preparations from non-failing and failing hearts was uniformly low (Figs. 1A and B). ANP increased GC activity similarly in membranes from non-failing and failing hearts. When fold activation was determined by dividing ANP-dependent activities by basal activities and compared between non-failing and failing samples, the values were identical (9.4-fold, data not shown). Thus, in contrast to heart-failed mice that have reduced ANP-dependent GC activity, we observed no difference in ANP-dependent GC activity measured in left ventricular samples from humans with or without heart failure.

CNP increased GC activity in the non-failing samples to 1.5 pmol cGMP/mg/min, which was 53±4% of the activity observed in the presence of ANP or 4.7-fold over basal activity. However, CNP stimulated GC activity was 2.0 pmol cGMP/mg/min in membranes from the failed ventricles, which represent a 6.3-fold stimulation and a 35% increase over values obtained in membranes from non-failing hearts. Thus, similar to activities measured in failing mouse ventricles, human ventricular CNP-dependent GC activity was about half that of the ANP-dependent activity; but in contrast to the mouse study, CNP-dependent GC activity was significantly elevated compared to non-failing values.

These data were then reanalyzed to determine what effect heart failure etiology has on natriuretic peptide dependent guanylyl cyclase activity (Fig. 1C). No difference was observed for the ANP-dependent activity between the three groups. Regarding CNP dependent activity, tissues from both ischemic and non-ischemic hearts exhibited an increase in CNP-dependent GC activity, but only the non-ischemic group was statistically significant ($p=0.006$).

### 3.3. ANP increases GC activity in cardiomyocytes from non-failed, but not failed ventricles

Recognizing that nonmyocytes may contribute to findings observed in membranes from crude tissue homogenates, we examined GC activity in membranes obtained from cell pellets that were highly enriched for cardiomyocytes [27]. Basal GC activities measured in membranes derived from myocyte-enriched preparations from non-failing hearts were loosely clustered and exhibited about 10% of the activity measured in the ventricular preparations when normalized to protein concentrations (Fig. 2). This indicates that non-cardiomyocyte cells express membrane guanylyl cyclases at higher levels than cardiomyocytes. Basal GC activities in the myocyte-enriched preparations from the failing hearts were even more widely dispersed and were not significantly different from activities

measured in equivalent preparations from non-failing hearts. Consistent with the increased basal values, GC activity determined in the presence of manganese and Triton X-100 was elevated, but not significantly, in cardiomyocyte pellet membranes from failed hearts.

ANP increased GC activity 2.4-fold in the pellet membranes from non-failing ventricles, whereas CNP only increased activity 1.3-fold over basal values (Fig. 2A). Neither ANP nor CNP significantly increased GC activity in pellet membranes from the failing ventricles. When the activity obtained in the presence of ANP or CNP was divided by basal activity and plotted as fold activation (Fig. 2C), it was clearly apparent that ANP-dependent GC-A activity was diminished in cardiomyocyte-enriched pellet membranes from failing ventricles compared to similar membrane preparations isolated from non-failing ventricles. Finally, similar to what was observed with the ventricular samples, separation of the failing samples into ischemic and non-ischemic groups indicated that CNP-dependent GC activity in the non-ischemic samples was higher than CNP-dependent activity from ischemic samples (Fig. 2D). These data indicate: 1) cardiomyocytes express much higher levels of GC-A than GC-B, 2) GC-A is expressed at reduced levels in cardiomyocytes compared to expression in other cells in the ventricle, and 3) that GC-A in the failing cardiomyocyte pellet preparation is refractory to ANP stimulation.

## 4. Discussion

Despite the clinical significance of natriuretic peptide receptor signaling in heart failure, very little information is available regarding the activation of natriuretic peptide receptors in human tissues. In fact, to our knowledge, this is the first report of CNP-dependent GC activity in any tissue and only the second report of ANP-dependent activity measured in human heart samples. We observed differential changes in ANP- and CNP-dependent GC activation in failing vs. non-failing ventricles. Furthermore, we implicate a far greater contribution of non-myocytes, rather than cardiomyocytes, to the GC activity in both failing and non-failing human hearts. Finally, we demonstrate that the small amount of GC-A in non-failing human cardiomyocytes is refractory to ANP stimulation in the failing heart.

In a previous study with a pressure-induced murine heart failure model, we observed that GC-A accounted for the majority of membrane GC activity in mouse ventricles and that GC-B activity was about 70% of the GC-A activity. However, a portion of the CNP-dependent activity was likely due to cross-activation of GC-A since 1μM CNP activates rat GC-A to about half of its maximum level [33]. We also observed that GC-A activity was reduced in hearts and kidneys of heart-failed mice and that GC-B was the most active natriuretic peptide receptor in failing murine ventricles due to GC-A downregulation [26,34]. The results showed a trend for increased GC-B activity in the failing mouse hearts, but it was not significant. In the current report, we measured natriuretic peptide dependent GC activity in human ventricular preparations and again found that GC-A was the primary membrane GC and that CNP stimulated cGMP levels to about half of the values observed with ANP. Unlike the rodent scenario where cross activation of natriuretic peptide receptors occurs, we can be certain that cGMP elevations resulting from CNP exposure are not due to activation of GC-A because CNP does not activate human GC-A at micromolar concentrations [33]. In contrast to the murine heart failure model, ANP-dependent activity was not reduced in

membranes from failing human ventricles. Additionally, CNP-dependent activity was significantly increased in failing human ventricles whereas it was only a trend in the mouse study.

We do not know if the disparate results concerning the ANP-dependent GC activity in the failing ventricles between mice and humans are a result of species differences or the increased variability (age, sex, ethnicity, etiology of failure, duration or severity of failure, etc.) of the human samples. After separation of the human failing samples based on etiology, the results were statistically similar to the pooled failing sample. However, with the separation, a trend toward a decrease in ANP-dependent GC activity was observed in the non-ischemic failing samples. The increase in CNP-dependent GC-B activity was also greater for both the ventricles and cardiomyocyte-enriched pellets of the non-ischemic patients, although increased activity in the pellet preparation appears non-specific since it was elevated under all assay conditions including basal. Different heart failure etiology may partially explain why our results differ from a previous study showing that ANP-dependent GC activity was reduced in failed human ventricles and that activity was restored when the hearts were unloaded [35]. In addition, in the previous study GC activity was poorly activated (<2.5 fold), whereas in our assays ANP stimulated GC-A 9-fold. These differences, as well as possible differences in tissue preparations and assay conditions of the two laboratories, specifically whether phosphatase inhibitors and EDTA were included in the buffers, may also explain the different ANP-dependent responses.

The current study extends the approach used in the previous murine study by evaluating GC activity of pellet preparations enriched in cardiomyocytes. Our results indicated that the natriuretic peptide receptor distribution in the pellet preparation was different from the intact ventricle in that GC-A expression was about ten-fold lower and GC-B activity was at the limit of detection. Thus, the increased GC-B activity observed in the ventricular preparation cannot be explained by increased GC-B activity in cardiomyocytes. Since GC-B is highly expressed in rat cardiac fibroblasts [36,37] and heart failure is associated with increased cardiac fibrosis [38], it is possible that the increased CNP-dependent GC activity detected in the failed ventricles results from an increased number of fibroblasts in the failing heart.

Our results showing increased GC activity in the myocyte-enriched pellets in the presence of ANP, but not CNP, suggest that cardiomyocytes express GC-A but much lower amounts of GC-B or no GC-B. This is an important, controversial, and currently unresolved issue. Conflicting reports have suggested that GC-B is or is not expressed on the surface of cardiomyocytes in rodents [25,36,37,39], but to our knowledge nothing has been reported concerning GC-B in human cardiomyocytes. While we measured a significant increase in GC activity with CNP, it was only increased1.3-fold over basal levels. Whether this slight increase results from very low expression in cardiomyocytes or contaminating fibroblast or other GC-B expressing cells is not known.

Finally, we do not know why basal GC activities were elevated in the pellet preparations from the failing hearts. One possibility is that endogenous ANP or BNP from the patient remained bound to the receptors during the membrane preparation. A previous cell culture studied demonstrated that ANP remains bound to GC-A despite multiple membrane washes

[31]. It is also possible that the increased activity is due to an increase in a GC besides GC-A, since activity determined in the presence of Triton X-100 and $Mn^{2+}$GTP was also slightly elevated.

In conclusion, we have shown that human heart failure is associated with increased ventricular CNP-dependent GC activity and that GC-A activity is severely blunted in cardiomyocytes from failing human hearts. Future experiments will seek to determine the ventricular cells types that express GC-B and to determine the molecular basis of GC-A inactivation in cardiomyocytes.

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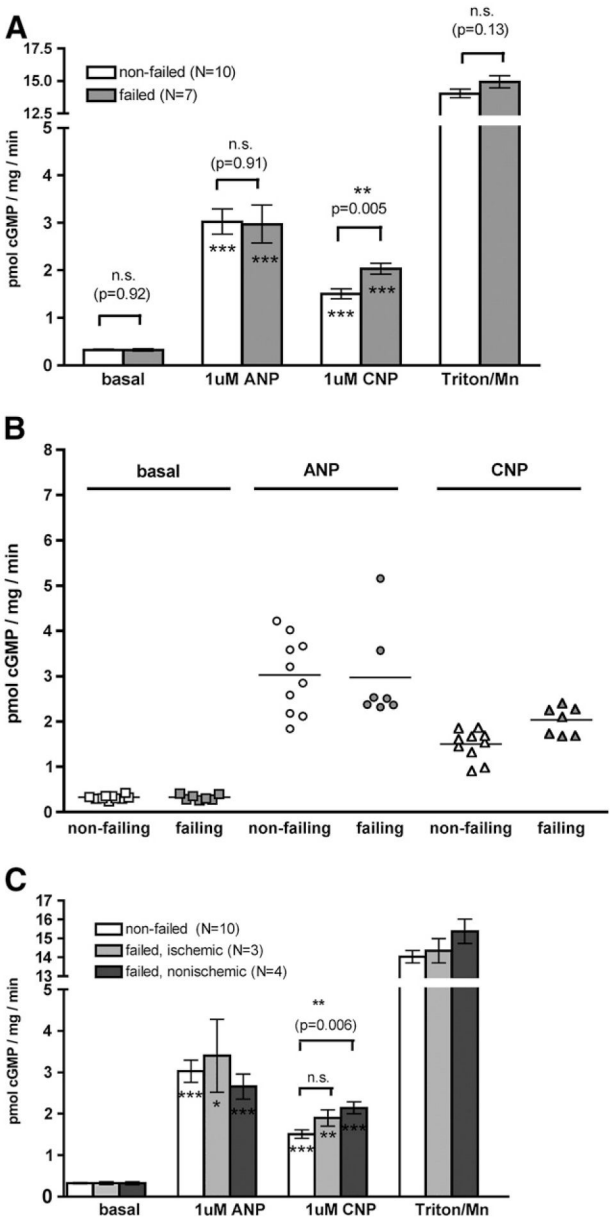


**Fig. 1.**

GC-B guanylyl cyclase activity is increased in left ventricle membranes from patients with heart failure. Membranes were prepared from ventricular tissue obtained from non-failed and failed human hearts. The membranes were assayed for guanylyl cyclase activity in the presence of 1 mM GTP, 1 mM ATP and $Mg^{2+}$GTP without (basal) or with 1 μM ANP or 1 μM CNP. Activity was also measured in the presence of 1% Triton X-100 where $Mn^{2+}$ was substituted for $Mg^{2+}$. (A) Mean guanylyl cyclase activity was plotted for each treatment. Statistically significant differences between basal activities and activities measured with ANP or CNP are indicated by including asterisks within the columns. The p values were ≤0.0001 for both sample sets. (B) A scatter plot of the guanylyl cyclase activities from individual samples is shown to demonstrate intragroup variability. (C) Separation of mean

guanylyl cyclase activity of failing samples from graph A based on heart failure etiology. Asterisks within the bars indicate statistical significance between basal and hormone-stimulated activities. P values are: *failed ischemic basal vs. ANP=0.025; **failed ischemic basal vs. CNP 0.0015; ***failed nonischemic basal vs. ANP 0.0003; ***failed nonischemic basal vs. CNP p≤0.0001.

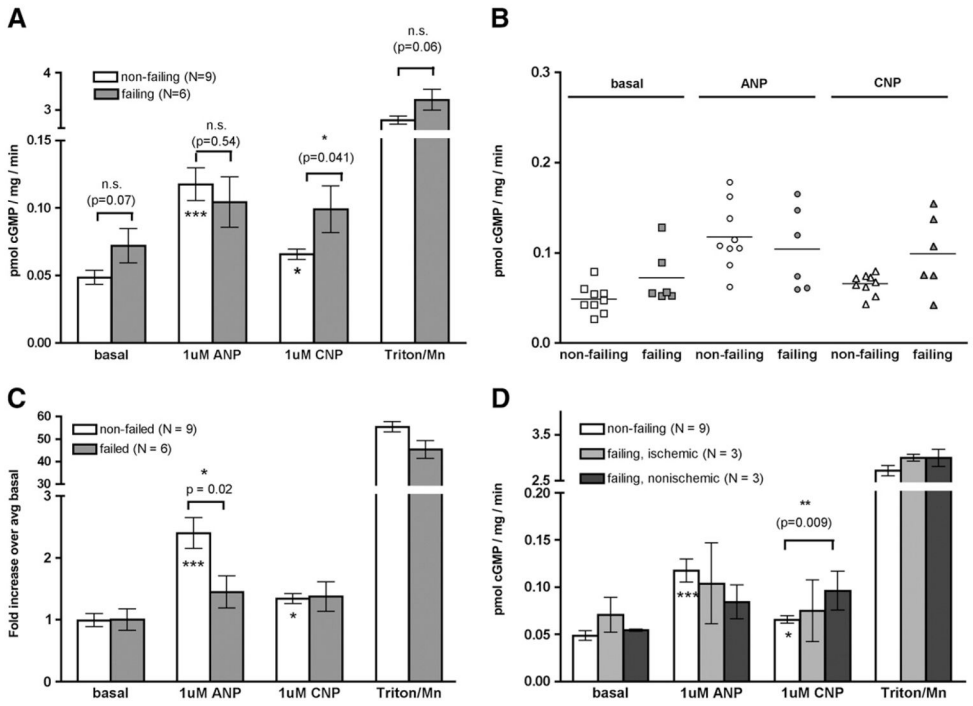


**Fig. 2.**

Enriched cardiomyocytes preferentially express GC-A. Membranes from enriched cardiomyocytes were assayed for guanylyl cyclase activity as described in Fig. 1. (A) Mean guanylyl cyclase activity is plotted for each treatment. Statistically significant increases over basal activity are indicated by asterisks within the bars. P values are: ***non-failed basal vs. ANP≤0.0001; *non-failed basal vs. CNP=0.018; failed basal vs. ANP=n.s. (0.18); failed basal vs. CNP=n.s. (0.24). (B) A scatter plot of the guanylyl cyclase activities from individual samples is shown to demonstrate intragroup variability. (C) Guanylyl cyclase activities from A are plotted as fold-increase over basal values. Statistically significant increases over basal are indicated by asterisks within the bars. P values are: ***non-failed basal vs. ANP ≤0.0001; *non-failed basal vs. CNP=0.018; failed basal vs. ANP=n.s. (0.18); failed basal vs. CNP=n.s. (0.24). (D) Separation of mean guanylyl cyclase activities from failing samples from graph A based on type of heart failure. None of the failed sample groups shows a statistically significant increase over basal.

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**Table 1**

Patient characteristics.

| | Age, y | Sex | Race | Body weight, kg | Heart weight, g |
|---|---|---|---|---|---|
| *Non-failing patients* | | | | | |
| 1 | 80 | Female | Caucasian | 73 | 367 |
| 2 | 28 | Male | Caucasian | 76 | 241 |
| 3 | 51 | Male | African-American | 91 | 407 |
| 4 | 65 | Female | Caucasian | 99 | 571 |
| 5 | 64 | Female | African-American | 73 | 459 |
| 6 | 43 | Male | Caucasian | 102 | 432 |
| 7 | 51 | Male | Hispanic | 68 | 322 |
| 8 | 56 | Male | Caucasian | 88 | 414 |
| 9 | 45 | Female | Caucasian | 75 | 338 |
| 10 | 61 | Male | Caucasian | 82 | 397 |
| *Failing patients* | | | | | |
| 1 | 47 | Male | Caucasian | 73 | 477 |
| 2 | 58 | Male | Caucasian | 73 | 399 |
| 3 | 20 | Male | Caucasian | 79 | 402 |
| 4 | 23 | Male | African-American | 122 | 361 |
| 5 | 40 | Female | Caucasian | 62 | 401 |
| 6 | 51 | Male | Hispanic | 102 | 389 |
| 7 | 21 | Female | Caucasian | 65 | 328 |

The general characteristics of the individual patients from which the ventricular tissue was obtained are shown.