The Aging Heart, Myocardial Fibrosis, and its Relationship to Circulating C-Type Natriuretic Peptide

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See Editorial Commentary, pp XX–XX

Abstract—Myocardial aging is characterized by left ventricular (LV) fibrosis leading to diastolic and systolic dysfunction. Studies have established the potent antifibrotic and antiproliferative properties of C-type natriuretic peptide (CNP); however, the relationship between circulating CNP, LV fibrosis, and associated changes in LV function with natural aging are undefined. Accordingly, we characterized the relationship of plasma CNP with LV fibrosis and function in 2-, 11-, and 20-month–old male Fischer rats. Further in vitro, we established the antiproliferative actions of CNP and the participation of the clearance receptor using adult human cardiac fibroblasts. Here we establish for the first time that a progressive decline in circulating CNP characterizes natural aging and is strongly associated with a reciprocal increase in LV fibrosis that precedes impairment of diastolic and systolic function. Additionally, we demonstrate in cultured adult human cardiac fibroblasts that the direct antiproliferative actions of high-dose CNP may involve a non-cGMP pathway via the clearance receptor. Together, these studies provide new insights into myocardial aging and the relationship to the antifibrotic and antiproliferative peptide CNP. (Hypertension. 2011;57:00-00.)

Key Words: aging ■ heart ■ C-type natriuretic peptide ■ fibrosis ■ natriuretic peptides

It is well established that myocardial aging is characterized by left ventricular (LV) fibrosis caused by the progressive reduction in cardiomyocyte number, the increase in cardiac fibroblast (CF) proliferation, and LV collagen deposition leading to ventricular dysfunction. To date, advances have been made in experimental and clinical studies in identifying humoral and hemodynamic mechanisms that contribute to age-related fibrosis in the heart. Such mechanisms include activation of cytokines such as transforming growth factor-beta and loss of nitric oxide (NO) together with LV pressure overload secondary to the rise in arterial pressure. C-type natriuretic peptide (CNP) is an antifibrotic and antiproliferative peptide that shares these key biological actions with the cardiac natriuretic peptides atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP). CNP is mainly an endothelial cell-derived peptide, which also has been detected in various other tissues including the heart and kidneys and circulates at low concentrations. Because of low circulating concentrations, CNP is thought to be mainly an autocrine/paracrine factor. Yet recently, CNP production has been identified in the human heart, liver, and brain with clearance in the lung and kidney, suggesting a role as an inhibitor of cardiac fibrosis. The relevance of CNP to fibrosis is compelling as CNP possesses potent antifibrotic properties through cGMP. Specifically in 1 key in vitro study, CNP possessed more potent antifibrotic and antiproliferative properties in young rat CFs compared to ANP and BNP. Furthermore, these investigators demonstrated in vivo that 14 days of continuous infusion of CNP in young rats with acute myocardial infarction (AMI) significantly attenuated post-AMI cardiac fibrosis. These 2 studies of CNP and its potential role as an inhibitor of cardiac fibrosis are complemented by reports that CNP possesses antifibrotic and extracellular matrix (ECM) regulatory actions in the lung, liver, and kidney. While Soeki et al and Horio et al demonstrated that CNP suppression of collagen production is linked to cGMP and the natriuretic peptide B receptor (NPR-B) in the young, evidence suggests that the natriuretic peptides may possess antiproliferative properties in part via activation of the non-cGMP natriuretic peptide clearance receptor (NPR-C), particularly in adults.

The current study tested the hypothesis that aging is characterized by a progressive decline in circulating CNP, which would be followed by a reciprocal increase in LV fibrosis compared to BNP. To address this hypothesis, we characterized circulating CNP in a Fischer rat model of aging, which is equivalent to human aging from adolescence to the sixth decade of life. We also determined LV structure, including LV fibrosis and function and defined the correlation of LV fibrosis to circulating CNP and BNP. Last, in vitro...
using adult human CFs, we tested the hypothesis that the antiproliferative action of CNP involves NPR-C.

Methods

Animals

Studies were performed in 2-, 11-, and 20-month–old male Fischer rats (Harlan Laboratories; n = 10 per age group, unless otherwise specified). These age groups are equivalent to human aging from adolescence to the sixth decade of life.20 The experimental study was performed in accordance with the Animal Welfare Act and with approval of the Mayo Clinic Institutional Animal Care and Use Committee.

Standard and 2-Dimensional Speckle-Derived Strain Echocardiography

Standard transthoracic echocardiography was performed on anesthetized (1.5% isoflurane in oxygen) rats using the Vivid 7 ultrasound system (GE Medical Systems) and a 10S transducer (11.5 MHz) with ECG monitoring. M-mode images and gray-scale 2-dimensional (2D) parasternal short axis images (300 to 350 frames per second) at the midpapillary level were recorded for off-line analysis using EchoPAC software (EchoPAC PC BTO 9.0; GE Healthcare). LV end-diastolic and end-systolic internal diameters and wall thicknesses were measured from M-mode images permitting calculation of LV ejection fraction (EF) based on the cubed method, LV fractional shortening (FS), and relative wall thickness (RWT). All parameters represent the average of 3 beats.

Two-dimensional speckle-derived strain echocardiography (2DSE) parasternal short axis images at the midpapillary level were acquired with a frame rate ranging from 60 (full apical views) and 160 (narrow sector views) frames per second as described previously.21 Three consecutive cardiac cycles were recorded as 2D cine loops, and the acquired raw data were saved for off-line analysis using EchoPAC software. Circumferential systolic peak values were determined for strain (sS) and strain rate (sSR). Circumferential early diastolic peak values were determined for myocardial strain rate (dSR-E).

Blood Pressure and Plasma Collection

Rats were anesthetized with isoflurane, and PE-50 tubing was placed into the carotid artery and placed in EDTA tubes on ice.7,22 Blood was immediately sampled. After BP acquisition using CardioSOFT Pro software (Sonometrics Corporation), blood was collected from the carotid artery and placed in EDTA tubes on ice.7,22 Blood was immediately centrifuged at 2500 rpm at 4°C for 10 minutes, and the plasma was stored in polystyrene tubes at −80°C for future radioimmunoassay.

LV Tissue Harvest

Hearts were removed for total cardiac and LV weights. The LV was dissected and quickly frozen in liquid nitrogen. A cross-section of the LV was preserved in 10% formalin for histological analysis, and smaller cube sections were preserved in 2.5% glutaraldehyde for electron microscopy (EM) analysis.

Plasma CNP and BNP Analysis

As described previously, plasma CNP was determined by a non-equilibrium radioimmunoassay kit (Phoenix Pharmaceutical), using an antibody to human CNP that is fully cross-reactive to rat CNP. One milliliter of plasma was extracted using C-18 Bond Elut cartridges. After washing cartridges with 4 mL of 100% methanol and 4 mL of water, plasma was applied, and the cartridges were washed. Eluates were concentrated on a Savant speed vacuum concentrator, and pellets were resuspended in 300 μL of assay buffer. One hundred microliters of standards and samples were incubated with 100 μL of antihuman CNP at 4°C. After 18 hours, 100 μL (10 000 counts) of 125I-labeled CNP were added and incubated at 4°C for 18 hours. Then a second antibody was added to all samples to separate the free and bound fractions, the samples were centrifuged, free fraction was aspirated, and bound fraction was counted on a gamma counter. A standard curve was generated, used to calculate the concentrations of the unknown samples, and reported in picograms per microliter. The range of the standard curve was 0.5 to 128 pg with a lower limit of detection of 0.5 pg. Interassay and intra-assay variability was 11% and 5.2%, respectively. Recovery was 72±6%. Cross-reactivity was <1% with ANP, BNP, endothelin, and adrenomedullin and 97% with CNP-53. Plasma BNP23 was also determined by nonequilibrium radioimmunoassay kits from Phoenix Pharmaceutical, using an antibody to rat BNP. All rat plasma samples were evaluated in 1 assay.

Histological and Electron Microscopy Analysis

For histology, fixed LV tissues were dehydrated, embedded in paraffin, and sectioned at a thickness of 4 μm. Collagen and extent of fibrosis was performed using picrosirius red staining (n = 7 per age group). An Axiosplan II KS 400 microscope (Carl Zeiss) was used to capture at least 4 randomly selected images from each slide using a ×20 objective, and KS 400 software was utilized to determine fibrotic area as a percentage of total tissue area. For EM, LV tissue fixed in 2.5% glutaraldehyde was dehydrated and embedded in a resin mold. Ultrathin sections were cut according to the EM core facility procedures. LV collagen fibers were visualized at ×8000 using a JEM-1400 transmission electron microscope.

Proliferation Assay

Seventy to 80% confluent passage 1 to 4 human adult CFs were serum starved for 24 hours and stimulated to proliferate using 10−8 M cardiotrophin-1 (CT-1) with or without 10−6 M BNP or CNP. For receptor antagonist studies, cells were treated with 10−6 M CNP with and without the nonselective NPR-A/B receptor antagonist HS-142-1 (10−6 M) or the selective NPR-C antagonist cANF 4−23 (des[Gln18, Ser19, Gly20, Leu21, Gly22] (ANP 4−23)-NH2) (10−6 M). Untreated CFs were processed as controls. Colorimetric bromodeoxyuridine (BrdU) cell proliferation enzyme-linked immunoadsorbent assay (Roche) was performed. CFs were labeled with BrdU for 2 hours in a CO2 37°C incubator. Anti-BrdU was added and allowed to react for 90 minutes at room temperature. The anti-BrdU was removed, and the CFs were washed 3 times with a washing solution. Colorimetric substrate solution was added, and color was allowed to develop for 30 minutes. Absorbance at 370 nm was measured on a SpectraMax spectrophotometer (Molecular Devices).

Table. Standard Cardiovascular Characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2 Months</th>
<th>11 Months</th>
<th>20 Months</th>
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<tr>
<td>BW, g</td>
<td>211±2</td>
<td>465±5*</td>
<td>445±7†</td>
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<tr>
<td>HW, mg</td>
<td>654±7</td>
<td>1149±23*</td>
<td>1089±29*</td>
</tr>
<tr>
<td>HW:BW, mg/g</td>
<td>3.11±0.03</td>
<td>2.47±0.03*</td>
<td>2.44±0.04*</td>
</tr>
<tr>
<td>LW, mg</td>
<td>472±6</td>
<td>829±15*</td>
<td>801±23*</td>
</tr>
<tr>
<td>LW:BW, mg/g</td>
<td>2.24±0.3</td>
<td>1.78±0.02*</td>
<td>1.80±0.03*</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>326±13</td>
<td>319±6</td>
<td>322±11</td>
</tr>
<tr>
<td>IVSd, mm</td>
<td>1.18±0.02</td>
<td>1.73±0.03*</td>
<td>1.65±0.06*</td>
</tr>
<tr>
<td>LVd, mm</td>
<td>6.71±0.10</td>
<td>7.62±0.06*</td>
<td>7.44±0.08*</td>
</tr>
<tr>
<td>LVWd, mm</td>
<td>1.21±0.03</td>
<td>1.69±0.2*</td>
<td>1.66±0.06*</td>
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<tr>
<td>EF, %</td>
<td>88±1</td>
<td>92±1*</td>
<td>80±1†</td>
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<tr>
<td>FS, %</td>
<td>51±1</td>
<td>58±2*</td>
<td>42±1*</td>
</tr>
<tr>
<td>RWT</td>
<td>0.36±0.01</td>
<td>0.45±0.01*</td>
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</tr>
<tr>
<td>MAP, mm Hg</td>
<td>91±1</td>
<td>91±2</td>
<td>102±4†</td>
</tr>
</tbody>
</table>

Values are mean±SEM. n = 10 for all age groups. *P<0.05 vs 2 months (1-way ANOVA), †P<0.05 vs 11 months (1-way ANOVA). HR indicates heart rate; IVSd, interventricular septum end-diastole; LVd, left ventricular internal diameter end-diastole; LVWd, left ventricular posterior wall end-diastole.
Statistical Analysis
Results are expressed as mean±SEM. Comparisons within groups were made by 1-way ANOVA followed by the Newman-Keuls multiple comparison test. A general linear regression model was used to test the correlation between LV interstitial fibrosis and plasma natriuretic peptides. GraphPad Prism software (GraphPad Software) was used for the above calculations. Statistical significance was accepted as \( P<0.05 \).

Results
Cardiac Structure and Function With Aging
Cardiac structure and function and mean arterial pressure (MAP) are reported in Table. There was a significant increase in body, heart, and LV weight (LVW); end-diastolic internal chamber diameter; and wall thicknesses and RWT at 11 months, which were sustained at 20 months. When heart weights (HW) and LVW were normalized to body weight (BW), there was a significant reduction in both HW:BW and LVW:BW ratios at 11 months, which was sustained at 20 months. Notably, LV EF and FS were significantly reduced at 20 months. At 20 months, there was a significant increase in MAP, and there was no change in heart rate among the groups. Furthermore, 2DSE confirmed a trend for LV functional impairment as demonstrated by reductions in circumferential sS (Figure 1A), sSR (Figure 1B), and dSR-E (Figure 1C) at 20 months compared to the younger age groups.

LV Interstitial Fibrosis
Figure 2 illustrates the quantification of picrosirius red staining (Figure 2A) and a representative image of LV interstitial fibrosis using picrosirius red staining (Figure 2B, top) and electron microscopy (Figure 2B, bottom) for each age group. Specifically, there was a significant and progressive increase in intensity of collagen staining (Figure 2A and 2B, top) and abundance in collagen fibers (Figure 2B, bottom, yellow arrows) between the age groups.

Plasma CNP and BNP and the Relationship to LV Fibrosis
Figure 3 illustrates plasma CNP and BNP with aging. Importantly, there was a significant progressive decrease in plasma CNP (Figure 3A) from 2 to 11 to 20 months of age (2-month mean±SE 31.4±3.6 pg/mL, 11-month mean±SE 20.6±0.8 pg/mL, 20-month mean±SE 9.0±0.4 pg/mL; \( P<0.0001 \)), whereas plasma BNP (Figure 3B) demonstrated a modest trend for an increase at 11 and 20 months (2-month mean±SE 21.0±2.1 pg/mL, 11-month mean±SE 25.1±1.3 pg/mL, 20-month mean±SE 26.5±1.6 pg/mL; \( P=0.08 \)). Furthermore, Figure 4A illustrates a strong negative correlation between LV interstitial fibrosis and plasma CNP (n=21; \( R^2=0.68; P<0.0001 \)). In contrast, plasma BNP did not correlate with LV interstitial fibrosis (Figure 4B; n=21; \( R^2=0.14; P=0.10 \)).
Antiproliferative Effects of CNP on Adult Human Cardiac Fibroblasts

Figure 5A illustrates the ability of CNP to significantly suppress cell proliferation in adult human CFs induced by CT-1 as assessed by BrdU uptake as a measure of DNA synthesis and cellular proliferation. Furthermore, we observed the antiproliferative response to CNP was significantly blocked by the NPR-C antagonist cANF(4–23), as illustrated in Figure 5B, with only a trend to suppress inhibition with blockade of NPR-B with HS-142-1.

Discussion

This is the first study to define the relationship between circulating CNP and myocardial fibrosis during natural aging using a Fischer rat model of aging. Specifically, we report that circulating CNP progressively declines with aging and that there is a highly significant inverse relationship between decreasing circulating CNP and increasing LV fibrosis. We also demonstrate that the antiproliferative action of CNP on adult human CFs involves NPR-C.

The first evidence of the biological importance of CNP was reported by Chusho et al., in which targeted disruption of the CNP gene in mice resulted in severe dwarfism attributed to impaired endochondral ossification. More recently, evidence has also shown that CNP has biological actions that go beyond skeletal remodeling and targets cardiac ECM regulation. Specifically, humans in their sixth decade of life with aortic stenosis are reported to have reduced CNP production in their aortic valves compared to young disease-free adults. Importantly, in vitro studies have demonstrated that CNP possesses more robust antifibrotic and antiproliferative properties in young rat CFs compared to ANP and BNP. Furthermore, other investigations demonstrated that a 2-week infusion of CNP or a CNP-based peptide suppressed post-AMI cardiac fibrosis. Together, these studies support an important cardio-protective role of CNP in inhibiting cardiac fibrosis and ECM remodeling.
In contrast to BNP, plasma CNP concentrations in the adult are low. Therefore, CNP has been thought to primarily be an autocrine/paracrine factor. However, recent studies suggest a possible endocrine role for CNP in adults, by demonstrating modest but significant increases in circulating CNP in HF and myocardial ischemia.\textsuperscript{11,27} In our study using a rat model of aging, we observed that aging is characterized by a progressive decline in circulating CNP. Our studies thus are, in part, consistent with the elegant human studies of Prickett et al,\textsuperscript{22} who reported that plasma NT-proCNP and CNP were high in children when skeletal growth and development are occurring and progressively declined with aging. Our findings of a decline in circulating CNP was in contrast to circulating BNP, which modestly increased with aging, as supported by previous studies.\textsuperscript{28} Additionally and consistent with our hypothesis, circulating CNP significantly and inversely correlated with LV interstitial fibrosis. While the current study supports the conclusion that circulating CNP declines with age in association with myocardial aging and fibrosis, review of the clinical literature is less clear in both health and disease. While the work of Prickett et al\textsuperscript{22} supports our observations, it is clear that especially in the presence of disease, circulating CNP may be elevated in adults including in the aged. Totsume et al\textsuperscript{29} reported plasma CNP to be increased in adults with chronic kidney disease; however, a correlation to age was not investigated. Moreover, in the presence of HF, Del Ry et al\textsuperscript{27,30} reported in key papers the elevation of plasma CNP with some association with aging, in which age was associated with higher CNP levels in adult HF subjects. Such an observation would be consistent with the report of Palmer et al\textsuperscript{11} in the presence of myocardial ischemia. Meanwhile, Gulberg et al\textsuperscript{31} reported a decrease in plasma CNP in adults with liver cirrhosis and normal renal function compared to controls; however, no correlation to age was noted. Based on these previous studies, a careful prospective study in humans from infancy to old age, especially without concomitant disease, is clearly warranted.

Aging is increasingly recognized as an endocrine deficiency state involving many biologically active molecules. Such deficiencies have been linked to senescent endothelial cell production of NO,\textsuperscript{32} impaired vitamin D synthesis in aging skin,\textsuperscript{33} and reduced growth hormone production.\textsuperscript{34} Here we demonstrate the progressive decline in circulating CNP with aging in an experimental model of aging. Although the mechanism of the reduction in CNP with aging was not a goal of the current study, additional studies both in animal models of aging and in humans should address 3 key mechanisms that include (1) diminished production or release of CNP from endothelial cells attributed to a reduction in endothelial cell number, (2) decreasing production of CNP in aging bone caused by cessation of skeletal growth, and (3) an increase in CNP degradation by neutral endopeptidase.

It is well known that aging is associated with LV fibrosis and altered function. However, as demonstrated here, the natural history of global LV functional impairment was not completely progressive compared to LV fibrosis. Interestingly, at 11 months we observed a significant reduction in circulating CNP together with a significant increase in LV fibrosis that preceded any significant alterations in LV function. Structurally, LVW plateaued at 11 months, as did LV chamber diameter and wall thickness. Importantly, LV systolic function, specifically LV EF, and circumferential sS and sSR were significantly reduced only at the more advanced age of 20 months, which is equivalent to the sixth decade of human life. In addition, impairment of LV diastolic function at 20 months was present as circumferential dSR-E was also significantly reduced. The mechanisms of this late reduction in LV function are likely multifactorial. First, the reduction in function may be related to increased LV fibrosis, but to a higher level that exceeded 11 months of age. This reduction in LV function would be secondary to accumulation of replacement fibrosis to preserve structural myocardial integrity caused by necrotic loss of cardiomyocytes\textsuperscript{35} and/or related to changes in collagen turnover attributed to an imbalance in procollagen biosynthesis, postsynthetic procollagen processing, and collagen degradation.\textsuperscript{36,37} In addition, the modest but significant increase in MAP at 20 months could also potentially contribute to increased LV fibrosis and impaired function. Nonetheless, this progressive increase in LV fibrosis, which is most likely to be high tensile strength type I collagen fibers,\textsuperscript{38} may ultimately impact diastolic and systolic function, particularly at the more advanced age of 20 months. Therefore, as age-related increases in myocardial fibrosis is a complex process involving multiple pathways and humoral factors in addition to CNP, additional studies are warranted to clarify these mechanisms.

To date, only 2 previous in vitro studies have reported the actions of CNP on CF proliferation and production of collagen. These studies demonstrate the ability of CNP to activate cGMP in young rat CFs, which may be linked to binding of CNP to NPR-B.\textsuperscript{12,14} Others report that CNP may have biological effects through the activation of the non-cGMP-linked receptor NPR-C,\textsuperscript{39,40} which originally was thought to function only as a clearance receptor. In our in vitro studies, pharmacological levels of CNP possessed potent antiproliferative actions on adult human CFs. Furthermore, the antiproliferative effect of CNP was attenuated by the NPR-C antagonist cANF(4–23), suggesting involvement of NPR-C. Such a concept of inhibiting proliferation of adult CFs by a natriuretic peptide via the activation of NPR-C is consistent with the report by Huntley et al,\textsuperscript{19} which demonstrated that the antiproliferative actions of BNP on adult human CFs was also attenuated by the NPR-C antagonist cANF(4–23). However, the antiproliferative actions of CNP involving NPR-C must be viewed with caution as high doses of CNP were used, without defining a dose-response relationship. Interestingly, Kaneki et al\textsuperscript{41} demonstrated that the signaling pathway for CNP regulation of bone formation in osteoblasts switches from the NPR-B/cGMP/protein kinase G pathway to the NPR-C/G\textsubscript{i} protein/phosphatidylinositol-specific phospholipase C pathway with aging, strengthening the concept of a functional role for NPR-C in adults. Indeed, a physiological role for NPR-C continues to grow with studies suggesting that NPR-C can hyperpolarize vascular smooth muscle cells, activate a nonskeletal cation current in CFs, diminish endothelial cell permeability, and exert antiproliferative actions on human CFs.\textsuperscript{19,39,40,42}
Study Limitations

Our study was designed to define for the first time the evolution of endogenous circulating CNP, a potent antifibrotic peptide, and its relationship to alterations in myocardial structure and function in an experimental model of aging. Additional studies are warranted in humans from childhood into advanced age, to establish whether the current findings are relevant to human aging. Moreover, the dose-response relationship of CNP and the antiproliferative effects on CFs needs to be defined in CFs in which NPR-C has been knocked down or in CFs harvested from mice in which NPR-C has been genetically deleted to strengthen the current findings. Additional studies are also needed in experimental aging to determine whether physiological and/or pharmacological replacement therapy with CNP can delay myocardial aging and LV fibrosis, advancing a role for CNP as a fibro-inhibiting therapeutic. Finally, it is important to keep in mind that LV fibrosis with aging is complex involving many humoral and mechanical mechanisms in addition to CNP.

Perspectives

Aging, in the absence of disease, is a complex phenomenon that may be associated with changes in LV structure including pronounced fibrosis and a subsequent decrease in LV function, increasing the risk of cardiovascular morbidity. This study demonstrates that a progressive decline in circulating CNP characterizes normal aging and is strongly associated with a reciprocal increase in LV fibrosis that precedes subsequent reductions in diastolic and systolic function. We also demonstrate using adult human CFs that the antiproliferative actions of high-dose CNP may involve a non-cGMP pathway via NPR-C. Thus, a relative decline in CNP bioavailability could be a contributor to age-related LV fibrosis. Furthermore, these findings underscore the potential of a CNP-based therapy for myocardial aging as well as pathophysiological conditions in which excessive LV fibrosis affects LV function.

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Disclosures

None.

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