Novel Role for the Potent Endogenous Inotrope Apelin in Human Cardiac Dysfunction

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Background—Apelin is among the most potent stimulators of cardiac contractility known. However, no physiological or pathological role for apelin–angiotensin receptor-like 1 (APJ) signaling has ever been described.

Methods and Results—We performed transcriptional profiling using a spotted cDNA microarray with 12 814 unique clones on paired samples of left ventricle obtained before and after placement of a left ventricular assist device in 11 patients. The significance analysis of microarrays and a novel rank consistency score designed to exploit the paired structure of the data confirmed that natriuretic peptides were among the most significantly downregulated genes after offloading. The most significantly upregulated gene was the G-protein–coupled receptor APJ, the specific receptor for apelin. We demonstrate here using immunoassay and immunohistochemical techniques that apelin is localized primarily in the endothelium of the coronary arteries and is found at a higher concentration in cardiac tissue after mechanical offloading. These findings imply an important paracrine signaling pathway in the heart. We additionally extend the clinical significance of this work by reporting for the first time circulating human apelin levels and demonstrating increases in the plasma level of apelin in patients with left ventricular dysfunction.

Conclusions—The apelin-APJ signaling pathway emerges as an important novel mediator of cardiovascular control.

(Circulation. 2003;108:r65-r72.)

Key Words: natriuretic peptides ■ heart failure ■ vasculature

Heart failure is a syndrome of chronic neuroendocrine activation precipitated by an inability of the heart to maintain perfusion of body tissues. Although study of individual signaling pathways at the genetic level has provided significant insights into this disease process, approaches such as gene knockout are limited by the interdependence of cellular systems. Simultaneous examination of the expression levels of thousands of genes with high throughput techniques such as microarray transcription profiling can be a productive strategy in polygenic diseases where complex interplay between gene and environment is prominent.1 Patterns of gene expression can be described and promising candidate genes identified for in-depth study.2 A few investigators have previously assessed gene expression using microarrays in human heart failure, comparing expression in diseased hearts with normal hearts or expression among hearts from patients with different diagnoses.2,3 One drawback of this approach, however, is the small number of unique samples studied compared with large interindividual variation in gene expression. Confounding factors such as age and sex are difficult to control with small sample numbers.4 Another approach that has recently been used by this laboratory and others is the comparison of heart tissue under decompensated conditions at the time of left ventricular assist device (LVAD) placement with tissue from the same heart under compensated conditions at the time of transplantation.5,6 Such paired samples allow a narrow focus on the changes that result from mechanical offloading of the failing ventricle and minimize the effect of interindividual variability. Several studies have previously established that functional changes characteristic of heart failure are reversed after offloading.7,8

The APJ receptor (alternatively known as angiotensin receptor-like 1) is 1 of a family of 7-transmembrane domain...
receptors first cloned in 1993. Although “orphan” for many years, the endogenous ligand was recently isolated and named apelin. Apelin and APJ are widely expressed in homogenates from rat and mouse organs in a pattern shared with angiotensinogen and the angiotensin receptor AT1, respectively. However, angiotensin II does not bind to APJ. In addition, whereas angiotensin is known to be a significant vasopressor, apelin reduces blood pressure via a nitric oxide–dependent, possibly central mechanism. Apelin is also thought to be involved in fluid balance. Finally, apelin was recently found to exert a stronger positive inotropic effect on a molar basis (EC$_{50}$ 33 pmol/L) than any agent yet described.

In this report of large-scale changes in gene expression after LVAD, we confirm the pathophysiologic importance of natriuretic peptides and uncover roles for many novel genes, including the G-protein–coupled receptor APJ. We demonstrate an increase in expression levels of APJ after mechanical offloading in heart failure and changes in both tissue and plasma levels of the APJ cognate ligand apelin. Localization of apelin to the vasculature in normal heart suggests a novel paracrine signaling pathway in the cardiovascular system.

**Methods**

This study was approved by the institutional review board of Stanford University. Informed consent was obtained from all participants.

**Implantation of Left Ventricular Assist Device**

The Novacor Left Ventricular Assist System (World Heart Corporation) was implanted, providing a left ventricular apical core (pre-LVAD). Left ventricular postimplantation tissue was obtained at cardiectomy. Normal left ventricular tissue was derived from a patient with no history of coronary disease or cardiomyopathy.

**RNA Isolation and Hybridization**

RNA isolation and hybridization were performed as previously described. Common reference RNA was Universal Pooled Human Reference RNA (Stratagene). Samples were hybridized to the Agilent Human 1 Catalog Array. A total of 44 hybridizations were performed on 11 pairs of pre- and post-LVAD RNA samples. Replicate hybridizations were performed as dye swaps.

**Scanning, Background Subtraction, and Normalization of Microarray Data**

Microarrays were scanned on an Agilent G2565AA Microarray Scanner System. Images were quantified using Agilent Feature Extraction Software (version A.6.1.1). Processing included local background subtraction and a rank consistency-based probe selection filter. Normalization was carried out using a LOWESS algorithm. Dye-normalized signals of Cy3 and Cy5 channels were used in calculating log ratios. Ratios were averaged for each dye swap using the arithmetic mean.

**Significance Analysis of Microarrays**

This algorithm has been described previously. Heat maps were generated with software written by the authors (see the Software link, reference 16). For each patient k, we calculated for every gene g the difference between averaged post-LVAD and averaged pre-LVAD expression levels. These differences are ranked within each patient in descending order. The rank of the gene g in patient k is denoted $R_{g,k}$. For every gene g, the rank consistency score $S_{g,m}$ is the normalized maximal (ie, the worst) rank of this gene among all patients, $S_{g,m} = \max_{1 \leq i \leq n} R_{g,i}/N$, where N is the total number of genes. Thus, a gene has a rank consistency score (RCoS) of s if and only if it ranks better than s in all n patients. Similarly, we can also compute the rank consistency score $S_{g,m}$ for m out of n patients. In this case, for each patient we rank genes as before. For each gene, we order its ranks, and then the score $S_{g,m}$ corresponds to the m-th best rank, as follows: $S_{g,m} = m$-th smallest $R_{g,i}/N$, $1 \leq i \leq n$.

To determine the statistical significance of this score, we compute $P$ values for all score levels, s. This is done under the null model of uniform and independent rank vectors. Therefore, for the m out of n RCoS, $P$ value(s) $= \sum_{s=0}^{m} \binom{n}{s} (1-s)^{n-s}$.

Using these $P$ values, we estimate false discovery and binomial surprise rates, comparing the observed number of genes with score s or better to the expected number of genes with such scores.

**Hierarchical Clustering**

Unsupervised, average-linkage, hierarchical clustering was performed using Cluster software and displayed with Treeview software.

**Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction**

Five genes were assessed in 7 individuals. After DNase treatment, cDNA was synthesized from 5 μg of RNA using MMLV reverse transcriptase (SuperScript II kit, Invitrogen). Amplification was carried out in triplicate at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. RNA quantity was expressed relative to 18S endogenous control. Fold differences were calculated by dividing the post-LVAD sample by the pre-LVAD sample. Linear regression was carried out using SPSS version 11.0.

**Apelin Assay**

Eight milligrams of tissue was boiled in 0.1 mol/L acetic acid for 10 minutes, homogenized, and then centrifuged at 12 000 rpm for 10 minutes, and the supernatant was used to quantify total protein concentration via the Bradford Assay (BioRad). Equal amounts of total protein (concentration 300 μg/mL) were used in the Apelin-12 ELA assay kit (Phoenix Pharmaceuticals) following manufacturer’s instructions. Plasma 50 μL was used directly for the assay. Comparisons were made using Student’s paired t test (tissue apelin levels) and one-way ANOVA with post hoc tests according to Fisher (plasma apelin levels) (SPSS software version 11.0).

**Immunohistochemistry**

Tissue was frozen in OCT (Tissue-Tek). Four-micron-thick sections were fixed in −20°C acetone and air-dried. Blocking was achieved using 10% goat serum (Zymed, South San Francisco, Calif). Sections were stained with apelin polyclonal antibody (Phoenix Pharmaceuticals, Belmont, Calif) and with CD31 (Cymbus Biotechnology Ltd, England). Secondary incubation used anti-rabbit envision (DAKO, England) and with CD31 (Cymbus Biotechnology Ltd, England). Secondary incubation used anti-rabbit envision (DAKO, England) and with CD31 (Cymbus Biotechnology Ltd, England). Sections were counterstained using hematoxylin.

**Results**

**Transcription Profiling Confirms the Importance of Recognized Markers of Left Ventricular Dysfunction**

We hybridized cDNA derived from paired samples of human left ventricle, harvested at the time of LVAD implantation and later at the time of cardiac transplantation, to 12 814 clone cDNA microarrays. Eleven patients were included in the study (Table 1). The number of genes significantly upregulated after mechanical offloading was greater than the
number downregulated (Figure 1A and Table 2; also see reference 16). At a false detection rate (FDR) of 0.05, Significance Analysis of Microarrays (SAM) identified 85 upregulated genes, whereas RCoS identified 82 genes, and 41 genes were identified by both algorithms. At an FDR of 0.05, SAM identified 13 downregulated genes, whereas RCoS identified 36 genes, and 10 downregulated genes were identified by both algorithms. Genes with reduced message identified 36 genes, and 10 downregulated genes were identified by both algorithms. Genes with reduced message included those coding known markers or marker precursors of heart failure such as natriuretic peptide precursor A (Unigene Hs.75640) and natriuretic peptide precursor B (Hs.219140). In addition, the natriuretic peptide features clustered together when subjected to average linkage hierarchical clustering (Figure 1B). These findings provide a unique validation for the use of natriuretic peptides as clinical markers through their emergence from a screening pool of many thousand genes. Additionally, they provide a rationale for our use of paired samples from offloaded hearts to characterize the genetic profile of heart failure.

**Novel Candidate Genes Emerge From Microarray Analysis**

The list of genes differentially regulated from pre- to post-LVAD contains genes previously unrecognized to be important in heart failure. Mitogen-activated protein kinase 4 (MAPK4, Hs.269222, also called ERK3, ERK4, and p63MAPK) is a distantly related member of the MAPK family of serine/threonine kinases. It was highly and consistently downregulated after LVAD and ranked ahead of all other genes by SAM (Figure 1A) and 10th by the RCoS method (Table 2). SAM ranks genes by a t statistic, which emphasizes overall pre- to post-differences per gene as a function of variance, controlling the error rate by a permutation procedure. In contrast, the rank consistency score more directly rewards consistency of change for a given gene across many individuals, offsetting the effect of individual variation.

Downregulation of a splice variant of the regulatory domain (α subunit) of the L-type calcium channel after offloading (AF233289, ranked 3rd by SAM) may be important given that changes in calcium dynamics are a central component of heart failure pathogenesis. Although the role of the myosin light chain kinase pseudogene (AF042089, 6th in rank consistency) remains unknown, myosin light chain kinase itself is a key mediator of sarcomeric organization in cardiac hypertrophy, making the presence of this related gene intriguing. Myosin light chain 2a (W17098, 5th in rank consistency) is a highly conserved and early marker of atrial chamber differentiation in organogenesis but is found here in the ventricle, suggesting a possible novel role in left ventricular hypertrophy and failure.

Several immunological markers, such as interleukins, interferons, tumor necrosis factor–related genes, and major histocompatibility complex genes, are upregulated after LVAD. Hierarchical average-linkage clustering (Figure 1B) groups these genes together, suggesting a coordinated immune response to the implantation of a prosthesis in the thorax.

**Gene Expression Does Not Predict Diagnostic Classification of Heart Failure**

Although some studies have compared gene expression in heart failure among different diagnostic categories, others contend there is a final common pathway regardless of initiating etiology. Hierarchical clustering of the entire data set lends limited support to this latter argument as patients do not cluster by diagnostic category (see reference 16). In addition, no genes were significantly differentially upregulated between diagnostic groups (SAM). Exclusion of 1 patient with hypertrophic cardiomyopathy and 1 with giant cell cardiomyopathy changed neither of these findings.

**Expression of the APJ Receptor Is Markedly Elevated After LVAD**

Two distinct statistical analyses identified APJ (Hs.9305) as the gene most significantly and consistently upregulated after LVAD implantation (Figure 1A, Table 2). The SAM score (4.872) was greater than all others, whereas pre- to post-LVAD fold change was estimated by hybridization at 3.2 and by quantitative real-time polymerase chain reaction (PCR) at 4.12. These relatively conservative fold changes contrast with the magnitude of the SAM score and emphasize the importance of variance in the analysis of microarray data. The number of significantly upregulated genes in this data set with fold changes less than 2 argues against the common practice of filtering on this criterion.

**Quantitative Real-Time PCR Confirms the Accuracy of Microarray Hybridization Studies**

Although use of a reference RNA controls much of the variation introduced by the dynamics of hybridization, the gold standard for quantitation of mRNA remains quantitative real-time (qRT) PCR (Taqman). We carried out qRT-PCR for 5 of the most differentially regulated genes in both samples of 7 individuals for whom sufficient RNA was available (Table 1) and expressed results as the ratio of post-LVAD to pre-LVAD for each individual and gene. The 5 genes were APJ, interleukin 6, MAPK4, atrial natriuretic peptide, and...
brain natriuretic peptide. We found a close relationship between the magnitude of change as measured by hybridization and qRT-PCR (Figure 1C; \( y = 0.49x \), \( R^2 = 0.86 \), \( P < 0.0001 \)), confirming previous authors’ observations and suggesting that ratiometric hybridization accurately reflects gene expression across the array.22

Apelin Is Increased in Cardiac Tissue After LVAD Implantation

We used competitive enzyme immunoassay to detect levels of the APJ ligand apelin in the samples of left ventricle that were used for hybridization. Tissue apelin levels were significantly higher after LVAD (Figure 2A; pre, 0.967 ± 0.26; post, 2.032 ± 0.56; \( P = 0.008 \)).

Figure 1. Microarray analysis of cardiac gene expression before and after insertion of a LVAD. A, Heatmap representation of genes significantly differentially regulated after LVAD. Rows represent individual genes; columns represent patients. Color intensity values are row-normalized, and rows are ordered according to the \( d \) statistic of the SAM. B, Dendrogram output of average-linkage hierarchical cluster analysis generated using Cluster and Treeview software.19 Genes from the significant gene list generated by SAM analysis at a false discovery rate of <5% cluster in functional groups such as immune markers and natriuretic peptides. Color saturation values represent absolute gene expression across all genes. C, Validation of microarray findings using qRT-PCR. Post- to pre-LVAD fold changes for 5 genes across 7 patients as determined by hybridization are plotted against those determined by qRT-PCR. Linear regression demonstrates a significant correlation (\( R^2 = 0.86 \)).
TABLE 2. Genes Significantly Differentially Regulated After Implantation of a LVAD

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<th>Gene Name</th>
<th>Accession No.</th>
<th>Rank Consistency</th>
<th>SAM Score</th>
<th>SAM Rank</th>
<th>Median Fold Change</th>
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<td>3.39</td>
<td>20</td>
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<td>Myosin light chain 2a</td>
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<td>211</td>
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<td>Myosin light chain kinase pseudogene</td>
<td>AF042089</td>
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The rank consistency score, here calculated for 9 out of 11 samples, rewards consistency of change in gene expression across individuals, offsetting patient-to-patient variation. The corresponding d statistic from the SAM is shown, along with the SAM list rank. Although variation is present in the ranking of genes identified by each method, APJ is the most significantly upregulated gene by both methods. Similarly, MAPK4 and natriuretic peptide genes are found near the top of the list of significantly downregulated genes by both methods. The upregulated gene list has been truncated.
post, 2.246±0.41 ng/mL; P<0.001; units are concentration of apelin in nanogram per milliliter within a normalized total protein concentration of 300 μg/mL). This reflects a change in the upward direction in all but 2 patients. Because the expression of the receptor and its ligand were moving in concert, we examined the relationship between the 2 per individual. We found a weak but significant positive correlation (y = −0.42 + 1.15x; R²=0.3; P=0.019; data not shown).

Apelin Is Highly Specifically Localized to the Vasculature in Cardiac Tissue
We carried out immunohistochemistry with the same antibody used for detecting apelin tissue levels by enzyme immunoassay. We compared the localization of apelin in normal human left ventricle with that from end stage, failing left ventricle. In both tissues, the distribution of staining was similar. We found that cardiac vessels stain densely for apelin with negligible staining in myocardial cells (Figure 2B). Staining of consecutive sections for platelet-endothelial cell adhesion molecule (CD31, middle) confirms the specificity of this localization. Control panels (left) represent sections where incubation in primary antibody was omitted.

Human Plasma Levels of Apelin Rise in Early Heart Failure and Fall in Severe Disease
To determine the role of apelin in earlier stages of heart failure, we measured plasma levels of apelin in blood from 80 heart failure patients with a broad spectrum of disease severity (male, n=63; female, n=17; mean age, 63 years; SD, 10 years). Because plasma apelin levels had not previously been reported in humans, we recruited 32 healthy subjects to determine the range of normal. We found that plasma apelin was detectable in plasma from healthy human subjects (3.58±0.33 ng/mL), rose in the early stages of heart failure (NYHA class 1, 4.94±0.85 ng/mL), and was maximum in those classified as NYHA class 2 (6.22±0.63, P<0.02). In those with severe disease, plasma apelin was lower (NYHA class 3 to 4, 4.58±0.62 ng/mL), but this change was not significant (Figure 3A). Mirroring the changes in functional class, dividing the patients by ejection fraction also revealed a rise in apelin from normal to mild-to-moderate left ventricular dysfunction (3.98±0.34 versus 6.02±0.72 ng/mL, P<0.02). Similarly, in later-stage disease, apelin level declined (severe left ventricular dysfunction, 4.11±0.58 ng/mL; P<0.02, Figure 3B).

Discussion
APJ is a 380-amino acid, 7-transmembrane domain G-coupled receptor most closely related to the angiotensin 2
The APJ cognate ligand apelin is secreted as a 77-amino acid preproprotein, and processed to 12-, 13-, and 36-amino acid moieties, which are variably produced in different tissues and show varying activity levels. Although expression of APJ and apelin has been characterized as widespread, studies at the cellular level have pointed to a localized signaling axis likely involved in specific aspects of cardiovascular development and function. In embryonic development, APJ expression is confined to the endothelium of the primary blood vessels and the newly forming heart, and apelin has been localized to sprouting vessels in the developing retina. Binding of radiolabeled apelin in the lung, where it is highly expressed, was concentrated in vascular tissue. Both APJ and apelin have been identified in regions of the hypothalamus involved in cardiovascular homeostasis, and indeed the apelin-APJ pathway has been implicated in drinking behavior and central control of blood pressure. In the heart, APJ has the same density as the angiotensin 2 receptor, and immunolocalization data reported here documenting apelin expression in the heart vasculature establish a paracrine signaling pathway in this organ. In tissue from patients with end-stage heart failure, apelin was also detectable in myocardial cells, suggesting an autocrine pathway of activation under diseased conditions.

After ventricular offloading, tissue levels of apelin protein and APJ message increase. Furthermore, although plasma levels of apelin rise in early heart failure, they fall in late disease. Szokodi et al infused apelin into isolated perfused rat hearts and demonstrated dose-dependent positive inotropy with preload-induced augmentation of the maximal rate of rise of left ventricular pressure. As such, it seems likely that along with other classical inotropic G-protein–coupled signaling pathways, the apelin-APJ system is recruited to support the contractility of the failing heart in mild-to-moderate left ventricular dysfunction. However, Tatemoto et al showed that administration of apelin peptides to anesthetized rats resulted in reductions in blood pressure of up to 26 mm Hg. This unusual combination of inotropy and afterload reduction suggests the apelin-APJ pathway as a therapeutic target in acute decompensated heart failure. In this situation, goals of therapy include diuresis, reduction of systemic vascular resistance, and support of contractility. We show here that not only is the apelin-APJ signaling pathway capable of inotropy and peripheral resistance reduction but that it is endogenously downregulated in severe disease. The idea that restoring levels of apelin in such patients could improve outcome remains to be tested. However, we hypothesize potential benefit from the exogenous administration of apelin in severe left ventricular failure, an idea supported by the increases in tissue protein levels of apelin and expression levels of the APJ receptor after LVAD demonstrated here.

Two recent studies using short oligonucleotide arrays have also assessed changes in gene expression after LVAD implantation. One study included 3 ischemic and 3 nonschematic cardiomyopathy patients and in contrast to our study found distinct gene expression differences between the 2 diagnostic categories. In a second study, an analysis of 7 patients with idiopathic dilated cardiomyopathy found a considerably different group of genes to be differentially regulated. In striking contrast to our findings, this second group found the APJ receptor to be downregulated by 3-fold and a generalized decrease in inflammatory gene signature after LVAD. The significant differences between these 3 array studies and the lack of correlation in some cases with previous studies at the individual gene level highlight the need to consider carefully the clinical features of the patients studied and to pursue at the biological level specific gene expression correlations.

Acknowledgments
This work was supported by the Donald W. Reynolds Cardiovascular Clinical Research Center at Stanford University.

References


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Circulation. published online September 8, 2003;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2003/09/08/01.CIR.0000091235.94914.75

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