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.

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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
eyears, the endogenous ligand was recently isolated and named
apelin. Apelin and APJ are widely expressed in homoge-
nates from rat and mouse organs in a pattern shared with
angiotensinogen and the angiotensin receptor AT1, respec-
tively. However, angiotensin II does not bind to APJ. In
addition, whereas angiotensin is known to be a significant
vasoconstrictor, apelin reduces blood pressure via a nitric oxide–
dependent, possibly central mechanism. Apelin is also
demonstrated to increase expression levels of APJ after mecha-
nical 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.

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
make a novel demonstration 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 Corpor-
ation) 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 Agi-
lent Human 1 Catalog Array. A total of 44 hybridizations were
performed on 11 pairs of pre- and post-LVAD RNA samples.

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).

Rank Consistency Score

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 descend-

ing order. The rank of the gene g in patient k is denoted Rg,k. For
every gene g, the rank consistency score Sg,k is the normalized
maximal (ie, the worst) rank of this gene among all patients,
Sg,k = \max_{1 \leq i \leq n} Rg,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
to better than s in all n patients. Similarly, we can also compute the rank
consistency score Sg,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 Sg,m corresponds to the m-th best rank, as follows:
Sg,m = m-th smallest Rg,k / N, 1 \leq k \leq n.

To determine the statistical significance of this score, we compute
P values for all score levels, s. This is done under the uniform model
of independent and rank vectors. Therefore, for the m out of n
RCoS, P value(s) = \sum_{s=0}^{m} C_{m}^{s} (1 - s)^{m-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 per-
formed using Cluster software and displayed with Treeview
software.

Quantitative Real-Time Reverse
Transcriptase–Polymerase Chain Reaction

Five genes were assessed in 7 individuals. After DNase treatment,
cDNA was synthesized from 5 \mu g 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 \mu g/mL) were used in the Apelin-12
ELA assay kit (Phoenix Pharmaceuticals) following manufacturer’s
instructions. Plasma 50 \mu L was used directly for the assay. Com-
parisons 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 Pharma-
cuticals, Belmont, Calif) and with CD31 (Cymbus Biotechnology Ltd,
England). Secondary incubation used anti-rabbit envision +
(DAKO, Carpenteria, Calif) for apelin and anti-mouse envision +
(DAKO) for CD31. The chromogenic substrate 3-amino-9-
ethylcarbazole was used. 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
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 (MAPK-4, 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.\(^1\) 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 number downregulated (Figure 1A and Table 2; also see reference 16).\(^1\) 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.

**TABLE 1. Clinical Characteristics of Patients Undergoing LVAD Implantation and Cardiac Transplantation**

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<thead>
<tr>
<th>Age, y/Gender</th>
<th>Diagnosis</th>
<th>Duration, d</th>
<th>qRT-PCR</th>
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<tr>
<td>63/M</td>
<td>ICM</td>
<td>170</td>
<td>+</td>
</tr>
<tr>
<td>17/M</td>
<td>IBCM</td>
<td>36</td>
<td>+</td>
</tr>
<tr>
<td>48/M</td>
<td>ICM</td>
<td>28</td>
<td>+</td>
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<tr>
<td>55/M</td>
<td>ICM</td>
<td>323</td>
<td></td>
</tr>
<tr>
<td>54/M</td>
<td>ICM</td>
<td>36</td>
<td>+</td>
</tr>
<tr>
<td>30/M</td>
<td>HCM</td>
<td>89</td>
<td>+</td>
</tr>
<tr>
<td>46/M</td>
<td>GCCM</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>18/M</td>
<td>ICM</td>
<td>92</td>
<td>+</td>
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</tbody>
</table>

ICM indicates ischemic cardiomyopathy; IBCM, idiopathic dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; and GCCM, giant cell cardiomyopathy. Duration indicates days to transplantation.

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
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.

**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 $d_i$ statistic of the SAM. B, Dendrogram output of average-linkage hierarchical cluster analysis generated using Cluster and Treeview software. 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 ($R^2 = 0.86$).

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, $0.967 \pm 0.26$; post, $2.44 \pm 0.70$; $P < 0.0001$).

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.
### TABLE 2. Genes Significantly Differentially Regulated After Implantation of a LVAD

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession No.</th>
<th>Rank Consistency</th>
<th>P Value</th>
<th>SAM Score</th>
<th>SAM Rank</th>
<th>Median Fold Change</th>
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<td>Angiotensin receptor-like 1</td>
<td>U03642</td>
<td>3.57183E-11</td>
<td>4.87</td>
<td>1</td>
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<td>Guanylate binding protein 1, interferon-inducible, 67 kD</td>
<td>M55542</td>
<td>1.00574E-09</td>
<td>3.25</td>
<td>26</td>
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<tr>
<td>P311 protein</td>
<td>NM_004772</td>
<td>1.00574E-09</td>
<td>3.49</td>
<td>12</td>
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<tr>
<td>Major histocompatibility complex, class II, DPα</td>
<td>X03100</td>
<td>1.85962E-08</td>
<td>3.39</td>
<td>20</td>
<td>2.46</td>
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<tr>
<td>Myosin light chain 2α</td>
<td>W17098</td>
<td>8.29795E-08</td>
<td>2.25</td>
<td>211</td>
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<tr>
<td>Myosin light chain kinase pseudogene</td>
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<td>3.72431E-07</td>
<td>3.49</td>
<td>12</td>
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<tr>
<td>Amylase, α2B; pancreatic</td>
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**Downregulated genes**

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<th>Gene Name</th>
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<th>SAM Score</th>
<th>SAM Rank</th>
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<td>NM_004317</td>
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<td>Human gene for natriuretic protein, partial cds</td>
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<td>Tec protein tyrosine kinase</td>
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<td>Aryl hydrocarbon receptor nuclear translocator</td>
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<td>Sushi-repeat protein</td>
<td>AF006567</td>
<td>8.66718E-06</td>
<td>−2.52</td>
<td>67</td>
<td>1.85</td>
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<td>Quiescin Q6</td>
<td>U97276</td>
<td>1.11165E-05</td>
<td>−1.71</td>
<td>450</td>
<td>1.64</td>
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<tr>
<td>Synovial sarcoma, translocated to X chromosome</td>
<td>NM_005637</td>
<td>1.21749E-05</td>
<td>−4.01</td>
<td>6</td>
<td>1.94</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.
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
receptor type 1. 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 and afterload-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 nonischemic 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.

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References


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