Clinical Research

Relationship of calcineurin expression between T-lymphocyte and myocardium in patients with heart failure

Yong Zhao¹, Jian-Chun Wang¹, Meng-Meng Wang¹, Chuan-Xia Wang¹, Wei Liu², Jian-Hua Shao¹

¹ Department of Geriatric Cardiology, Shandong Provincial Hospital, Jinan 250021, China
² Operating Room, Shandong Provincial Hospital, Jinan 250021, China

Objective Congestive heart failure (CHF) is the final common pathway of various heart diseases. Calcineurin, a calcium/calmodulin-dependent phosphatase consisting of a catalytic subunit A (CnA) and a regulatory calcium-binding subunit B (CnB), is activated in heart failure. This study aimed to investigate the relationship between mRNA level of calcineurin in circulating T-lymphocyte and that in myocardium in patients with CHF. Methods A total of 38 patients with CHF (aged from 29 to 62 years) were included in this study. The mRNA levels of alpha- and beta-isoform of CnA in left ventricular anterior papillary muscle and peripheral lymphocytes were determined by semi-quantitative reverse transcription polymerase chain reaction. Pearson linear correlation analysis was performed, and difference was considered statistically significant at a P value <0.05. Results Calcineurin mRNA levels in lymphocytes were positively correlated with those in myocardium (for CnA-alpha mRNA, r=0.820; for CnA-beta mRNA, r=0.875; both P<0.01). CnA-beta mRNA levels in both circulating lymphocytes and myocardium increased significantly with increasing NYHA class (r=0.877 for peripheral blood and r=0.805 for cardiac muscle; both P<0.01). Conclusions The mRNA level of CnA-beta in circulating lymphocytes is positively correlated with that in myocardium and is a promising marker for the severity of cardiac dysfunction in patients with CHF (J Geriatr Cardiol 2010; 7:93-96).

Key words calcineurin; heart failure; lymphocytes; myocardium

Introduction

Congestive heart failure (CHF) is the final common pathway of various heart diseases and is often preceded by a period of myocardial hypertrophy. CHF confers a substantive burden on public health, and 78% of patients with CHF have left ventricular systolic dysfunction.¹ Although the strategies of pharmacological intervention have been dramatically changed in recent years with the recognition of neurohumoral activation in the pathophysiology of CHF, the underlying molecular mechanism is not fully elucidated yet. Accumulated experimental evidences suggest that many signaling pathways may be implicated in the process of CHF, such as mitogen-activated protein kinases (MAPKs), protein phosphatase, calcineurin (CaN), and protein kinase Akt.²

CaN is a highly conserved calcium/calmodulin-dependent heterodimeric serine/threonine protein phosphatase, consisting of a catalytic subunit, calcineurin A (CnA), and a regulatory calcium-binding subunit, calcineurin B (CnB). Up to date, three isoforms of CnA and two isoforms of CnB have been identified. They are named CnAα, CnAβ, CnAγ and CnB1, CnB2 respectively. Except that CnAγ is exclusively expressed in testis and interacts with testis-specific CnB1, both CnAα and CnAβ are widely distributed in the most mammalian tissues with the highest levels in brain and bind to the common regulatory subunit, CnB2.³ It is well known that calcineurin regulates various cellular processes, including T-lymphocyte activation and cardiac remodeling.³ This study aimed to investigate the relationship between the mRNA level of calcineurin in circulating T-lymphocyte and that in the myocardium in patients with CHF, as well as the relationship between calcineurin expression level and the severity of CHF.

Methods

Patients and samples

The study was approved by the Ethic Review Committee of Shandong Provincial Hospital, Shandong University, and was compliant with Helsinki Declaration. Written informed consent was obtained from each participant.
Thirty-eight patients with CHF who underwent cardiac surgery for mitral valvular disease were recruited from January to August, 2007. The participants consisted of 20 males and 18 females with average age of 45 ± 8 years (29-62 years). Patients were classified as New York Heart Association (NYHA) classes II (n=11), III (n=15) and IV (n=12).

Venous blood sample (6 ml) was collected from each patient at hospitalization and mixed with anticoagulant ethylene diamine tetraacetic acid (EDTA). Phosphate buffer solution (PBS, 0.02 mmol/L, pH = 7.4) and lymphocyte separation medium (LSM, Shanghai Generay Biotech Co., Ltd. China) were added to harvest lymphocytes (the volume ratio of blood sample, PBS and LSM was 3 : 3 : 5). Centrifuged at 2 400 r/min for 25 min, blood cells between upper and middle layers were sucked out and washed twice with 5 ml PBS. After another centrifugation at 1 800 r/min for 7 min, lymphocytes were obtained, and then they were transferred into Eppendorff tubes, which had been treated with diethylpyrocarbonate (DEPC). Several pieces of papillary muscles were cut off from left ventricle during operation and were stored at -80°C. Total RNA in both lymphocytes and papillary muscles were extracted for analysis of CaN mRNA expression.

**Laboratory procedures**

Total RNA was isolated from lymphocytes and papillary muscles using single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction. The purity and concentration of total RNA were evaluated by ultraviolet visible light spectrophotometer with OD$_{260}$/OD$_{280}$ > 1.8.

The secondary conformation of RNA was destructed at 70°C for 5 min, then cDNA was synthesized by reverse transcription (RT) in a total volume of 20 μl at 37°C for 1 hr, and finally this process was terminated at 70°C for 10 min. After quantitative analysis, the cDNA of each patient was diluted by DEPC to the same concentration with the specimen of the minimal level as standard.

The cDNAs of CaN α, CaN β and internal reference β-actin had been replicated respectively by polymerase chain reaction (PCR) with their corresponding primers as following:

**CnA α**
- forward: 5’-GGGCAACCTCAGTCTTTG-3’
- reverse: 5’-TTTTTGACCTGATACGT-3’

**CnA β**
- forward: 5’-CTGACTCCACAGGAGATTG-3’
- reverse: 5’-GTGGTTCTCAGTGGCATG-3’
- β-actin
- forward: 5’-ACACTGTGCCCATCTACGAGGG-3’
- reverse: 5’-ATGAGTGAAGCTAGTTCCGATG-3’

Pre-denaturation was performed for 5 min at 95°C, followed by 30 PCR cycles with denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 45 sec. This reaction was terminated at 4°C for 10 min.

The amplification product of CnA α, CnA β and β-actin was 142 bp, 246 bp and 366 bp, respectively. Electrophoretic mobility shift assay was performed on 1.6% agarose gel, and photograph was taken under ultraviolet ray. The integral optical density (iOD) of each electrophoretic band was estimated with picture analysis, and the iOD ratios of CnA α and CnA β in both lymphocytes and myocardium to β-actin were calculated, respectively.

**Statistical analysis**

The continuous variables were presented as means ± SD. Differences among groups with different NYHA class were tested with ANOVA. Relationship between the mRNA level of calcineurin in circulating T-lymphocytes and that in myocardium was analyzed with Pearson linear correlation, and the relationship between calcineurin expression level and the NYHA class was analyzed with Spearman rank correlation. Statistical analysis was performed using SPSS 11.5 statistic package. Statistical significance was considered at a P value < 0.05.

**Results**

**mRNA expression of CaN**

Both CnA α and CnA β were expressed in circulating T-lymphocytes and myocardium (Fig. 1). The mRNA levels of both, CnA α and CnA β, especially CnA β, were increased with increasing NYHA class of cardiac function (Table 1). The expression of CnA β was significantly different between any two groups of NYHA II, III, and IV, with the highest in NYHA IV and the lowest in NYHA II patients (P<0.05).

**Correlation analysis of CaN mRNA**

The mRNA expression levels of both CnA α and CnA β in circulating lymphocytes were positively related to their corresponding levels in myocardium (Fig.2 and Table 2). The mRNA expression of CnA β, but not CnA α, in both circulating lymphocytes and myocardium was positively associated with NYHA class (Table 2). No statistical correlation was found between the expression of both CnA α and

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**Fig.1** Expression of CnA α and CnA β in circulating lymphocytes and myocardium
CnA β in either circulating lymphocytes or myocardium and the age or gender of the patients (Table 2).

Discussion

Intracellular Ca$^{2+}$ plays a pivotal role in the regulation of myocardial remodeling and cardiac function. The alteration of Ca$^{2+}$ homeostasis may result in myocardial hypertrophy and heart failure. CaN is a Ca$^{2+}$-activated phosphatase. Studies in recent years showed that the regulatory activity of CaN-nuclear factor of activated T cells (CaN-NFAT) pathway in myocardium is dramatically increased in patients with CHF, and Choudhary et al found a positive correlation between serum CaN activity and left ventricular hypertrophy. The present study not only confirmed the above observations by showing that the mRNA expression of CnA β, rather than CnA α, in both circulating lymphocytes and myocardium was positively associated with NYHA class of cardiac function, but also demonstrated a significant correlation between mRNAs expression of CnA α and CnA β in circulating lymphocytes and those in myocardium. The more severe the cardiac function is, the more humoral factors are

**Table 1** mRNA expression of CaN in both circulating lymphocytes and myocardium (IOD ratio of CaN to β-actin, $\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>NYHA class of cardiac function</th>
<th>n</th>
<th>Circulating lymphocytes</th>
<th>Myocardium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CnA α</td>
<td>CnA β</td>
</tr>
<tr>
<td>II</td>
<td>11</td>
<td>0.682 ± 0.131</td>
<td>0.599 ± 0.029</td>
</tr>
<tr>
<td>III</td>
<td>15</td>
<td>0.761 ± 0.175</td>
<td>0.655 ± 0.073</td>
</tr>
<tr>
<td>VI</td>
<td>12</td>
<td>0.705 ± 0.159</td>
<td>0.906 ± 0.063</td>
</tr>
</tbody>
</table>

**Table 2** Correlation coefficient of CaN mRNA in circulating lymphocytes and myocardium with cardiac function, age, and gender of patients

<table>
<thead>
<tr>
<th></th>
<th>CnA α in lymphocytes</th>
<th>CnA β in lymphocytes</th>
<th>NYHA class of cardiac function</th>
<th>Age</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>CnA α in lymphocytes</td>
<td>-</td>
<td>-</td>
<td>0.064*</td>
<td>0.516*</td>
<td>0.033*</td>
</tr>
<tr>
<td>CnA β in lymphocytes</td>
<td>-</td>
<td>-</td>
<td>0.877**</td>
<td>0.760*</td>
<td>0.064*</td>
</tr>
<tr>
<td>CnA α in myocardium</td>
<td>0.820**</td>
<td>-</td>
<td>0.092*</td>
<td>0.742*</td>
<td>0.019*</td>
</tr>
<tr>
<td>CnA β in myocardium</td>
<td>-</td>
<td>0.875**</td>
<td>0.805**</td>
<td>0.019*</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01

**Fig. 2** Correlation between the mRNA expression of CnA β in circulating lymphocytes and that in myocardium

Discussion

Intracellular Ca$^{2+}$ plays a pivotal role in the regulation of myocardial remodeling and cardiac function. The alteration of Ca$^{2+}$ homeostasis may result in myocardial hypertrophy and heart failure. CaN is a Ca$^{2+}$-activated phosphatase. Studies in recent years showed that the regulatory activity of CaN-nuclear factor of activated T cells (CaN-NFAT) pathway in myocardium is dramatically increased in patients with CHF, and Choudhary et al found a positive correlation between serum CaN activity and left ventricular hypertrophy. The present study not only confirmed the above observations by showing that the mRNA expression of CnA β, rather than CnA α, in both circulating lymphocytes and myocardium was positively associated with NYHA class of cardiac function, but also demonstrated a significant correlation between mRNAs expression of CnA α and CnA β in circulating lymphocytes and those in myocardium. The underlying mechanism accounting for these correlations is not well defined yet. We hypothesize that the local and systemic neurohormones, such as angiotensin II, endothelin-1 and urotensin II, which are activated during CHF, stimulate the elevation of free calcium ion (Ca$^{2+}$) in cytoplasm. The binding of increased Ca$^{2+}$ to the regulatory subunit CnB of CaN induces conformation changes and dislocation of the autoinhibitory domain in CnA. The active site of its phosphatase is then exposed and the CaN is activated. Subsequently, the component of down-stream signaling, NFATc2 transcription factor, is dephosphorylated and transfers into nucleus to modulate gene expression in cells with the synergetic action of other transcription factors, including MEF2, GATA4 and AP-1, which results in the increased mRNA expression of CnA α and CnA β in both circulating lymphocytes and myocardium. The more severe the cardiac function is, the more humoral factors are
activated, and consequently the more mRNA of CaN is expressed.

The changes of CnA α and CnA β were not parallel in response to CHF, in which the mRNA expression of CnA β was more closely associated with the severity of cardiac function than that of CnA α. An autopsy observation conducted by Grammer and his colleagues showed that, even in patients with aortic stenosis who had normal ejection fraction and left ventricular hypertrophy, a pathological condition preceding heart failure, the mRNA level of CnA- β, instead of CnA α, in myocardium was significantly elevated in comparison with normal myocardium, while the activity of CaN was not considerably different between them. These data suggest that CnA β play an important role in the development of CHF mechanically.

The analysis of CaN in myocardium is not convenient for evaluating heart failure clinical practice due to the difficulty of access to the heart specimen. The present study illustrated that CaN transcription, especially the mRNA of CnA- β, in circulating lymphocytes was positively correlated with that in myocardium and the severity of cardiac function, which implies that the assessment of CnA β mRNA expression in circulating lymphocytes should be a favorable approach to investigating the molecular mechanism of CHF or appraising the long-term efficacy of neurohormonal antagonistic therapy for CHF patients.

References