Laboratory Research

Valsartan prevents the development of rabbit's heart failure by restoring calcium uptake of sarcoplasmic reticulum

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Objective Clinical evidence has suggested that AT1 receptor blocker (ARB) could prevent the development of heart failure. Decreased sarcoplasmic reticulum(SR) Ca^{2+} content, which is due to reduced SR calcium reuptake by SERCA2a, is responsible for defective systolic function in failing heart. To better understand how ARB could improve cardiac systolic dysfunction, we studied the effects of Valsartan on calcium reuptake of SR and its regulatory proteins in heart failure rabbits. **Methods** Thirty rabbits were divided into three groups: sham rabbits(controls, n=11), rabbits with heart failure treated with Valsartan (n=11) and rabbits with heart failure but without Valsartan treatment (n=8).Rabbit heart failure model was established by volume plus pressure overload. Cardiac function was measured by echocardiography. SR calcium uptake was determined by measuring extra vesicular free [Ca^{2+}] changes in a fluorescence spectrophotometer. SERCA2a, Ser16-phosphorylated phospholamban (p-PLB), PKA and PP1á protein abundance were determined by use of Western blot analysis. **Results** Compared to control rabbits, the ejection fractions in the HF rabbits were significantly decreased (P<0.05), these changes could be significantly attenuated by Valsartan treatment (P<0.05).Calcium reuptake of SR, activity of SERCA2a and PKA decreased in heart failing myocytes (P<0.05), with down regulations of p-PLB, SERCA2a and PKA, but up regulation of PP1α in ventricular samples from the failing rabbits (P<0.05). All of these changes were attenuated by Valsartan treatment (all P<0.05). **Conclusion** Valsartan improved cardiac function in volume plus pressure overload induced heart failure of rabbits possibly by restoring the SR calcium uptake resulted from attenuating the activities and expressions of SERCA2a and its regulatory proteins **(*J Geriatr Cardiol* 2009; 6:173-177)** .

Key words calcium; heart failure; sarcoplasmic reticulum; Valsartan

Introduction

Contraction of human cardiac myocytes is a Ca^{2+} dependent process. The depressed contractility of the failing heart may be due to problems of Ca^{2+} handling by the sarcoplasmic reticulum (SR), the main source of Ca^{2+} for cardiac contraction.[1] Decreased SR Ca^{2+} content, which is due to reduced SR calcium reuptake by SERCA2a, is responsible for defective systolic function in failing heart,[2,3] but still conversely.[4] The SERCA2a activity is regulated by phospholamban (PLB) and other regulatory proteins including PKA and PP1α etc. Although alterations in SERCA2a and PLB have been reported, these changes have not been uniformed observed in failing heart.[5-7]

Clinical evidence has suggested that AT1 receptor blocker could prevent the development of heart failure.[8] However, except for that it reverses ventricular remodeling, it is not clear how can it improve cardiac systolic dysfunction. Therefore, the aim of this study was to investigate whether there were changes of SR Ca^{2+} reuptake, and then determine the expression and activity of SERCA2a and its regulatory proteins in rabbit failing myocytes. Furthermore, we observed the effect of Valsartan on the SR Ca^{2+} reuptake of failing heart.

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Methods

Heart failure model

We used 30 rabbits (weight 2.0-2.5 kg, either sex) approved by the Animal Ethics Committee of Suzhou University. All surgical procedures were performed in sterile conditions after anesthesia with 3% pentobarbital sodium (30 mg/kg) while the animals were spontaneously breathing room air. Chronic heart failure was induced by combined chronic volume and pressure overload.[9] Aortic insufficiency was induced by introducing a 4F catheter into the carotid artery and forcing it into the aortic sigmoid valves. Aortic pulse pressure (APP=systolic minus diastolic aortic blood pressure) was determined before (APPb) and after (APPa) the procedure. The APPa/APPb ratio had to be more

than 1.50. Two weeks after the first surgical procedure, the abdominal aorta was exposed, and a 5F catheter was positioned alongside it. The catheter and aorta were ligated together just above the right renal artery, and the catheter was then gently removed, thereby reducing the abdominal aortic lumen by about 50%. At the end of the study, the cardiac structure and function were measured by echocardiography (Sonos 7500 type Doppler Ultrasound, Siemens, German). Control animals (C; *n* =11) underwent the same surgical operations but without induction of aortic insufficiency and constriction. The failing rabbits with Valsartan treatment (HF with Valsartan, *n*=8) were administered by Valsartan with 20mg/kg/day after the first surgical procedure. The left rabbits were not treated with Valsartan (HF, *n*=11).

Isolation of SR vesicles

The left ventricle was finely minced and homogenized in four volumes of ice-cold homogenization buffer (mmol/L: 300 sucrose, 20 MOPS adjusted with Tris base to pH 7.2,2 DTT, 3 $MgCl_2$) and a combination of protease inhibitors (4 μg/ml Leupeptin, 4 μg/ml Pepstatin A, 1 mmol/L Benzamidine, 1 mM phenylmethylsulfonyl fluoride). Tissues were homogenized in a homogenizer using three 15 s bursts at 24,000 rpm followed each time by 5 s rest intervals. The homogenate was sedimented at 3800 × g for 20 min and the supernatants were filtered through cheesecloth and were sedimented at 14,000 × g for 20 min. Then, the supernatants were sedimented at 45,000 × g for 60 min. The sendiment was resuspended in homogenization buffer (mmol/L: 300 sucrose, 20 MOPS adjusted with Tris base to pH 7.0, 2 DTT, 0.1 mmol/L PMSF, 0.5 KCl) and sedimented at 45,000 × g for 60 min.The final sendiment, enriched in SR vesicles, was resuspended in homogenization buffer (in mmol/L: 300 sucrose, 20 MOPS adjusted with Tris base to pH 7.0, 2 DTT, 0.1 mmol/L PMSF), fractionated in small aliquots and snap-frozen in liquid N_2. Vesicles were stored at -80°C for up to 2-3 weeks.[10-11]

SR Ca^{2+} uptake assay

SR vesicles were first diluted to a total protein concentration of 5μmg/ml in 2 ml uptake buffer containing (mM: 100 KCL, 5 $MgCl_2$, 20 Tris-HCl, 0.1$CaCl_2$, pH 6.8) with 2μg/ml Fura-2(from Alexis) for 15 min at room temperature. Calcium uptake was determined at room temperature by measuring extra vesicular free [Ca^{2+}] changes in a fluorescence spectrophotometer (LAMBDA DG-4, USA). Uptake was initiated by the addition of 4μm/l Na-ATP. Fura 2 was excited at 340- and 380-nm wavelengths, and fluorescence emission was measured through a 510-nm band-pass filter (10-nm bandwidth). Fluorescence ratios (340-380nm) were obtained every 5s. The Ca^{2+} concentration corresponding to a given Fura-2 fluorescence ratio was estimated by applying the following equation :$[Ca^{2+}]=K_d \cdot \beta \cdot (R-Rmin)/(Rmax-R)$. Where Rmin and Rmax are the 340nm/380nm fluorescence ratios for saturation and zero Ca^{2+} solutions; K_d is the dissociated constant of Ca^{2+} as 0.144μm/l; β is the ratio of the emitted fluorescence at 380nm excitation in the Ca^{2+} free solution over that at 380nm in the saturation solution; and R is the observed 340nm/380nm fluorescence ratio.

Measurement of SERCA2a and PKA activity

Left ventricular tissue samples were cut into pieces and placed in the tubes with lysis buffer and protease inhibitors on ice. The lysate was homogenized and then centrifuged at 12000r/min at 4°C for 1 hour. The sendiment was resuspended in the same solution, and frozen for subsequent analysis. The protein concentration was assayed by Bradford. The measurement of SERCA2a and PKA activity were carried out strictly according to instruction manual from Nanjing Jiancheng Bioengineering Institute (China) and Preomega (USA) respectively. The activity of the Ca^{2+}-ATPase was given in micromoles of ATP per milligram of protein per hour. The activities of PKA were measured by using liquid scintillation counter and expressed as pmol value of 32P in histone catalyzed by per ìg protein per min.

Western blot analysis

Total protein was separated by SDS-PAGE (Power/PAC 300, Bio-RAD, USA). The samples containing 20ug total protein were first added to the sample buffer and boiled at 100°C for 5 min, and then applied to polyacrylamide gradient gel (SERCA2a, from ABR; PKA, from Abcam; PP1 α, from upstatebiotech; p-PLB, from upstate biotech). After electrophoretic separation at 100 V for 80 minutes in Tris-glycine/SDS buffer, proteins were transferred to nitrocellulose membranes by running for 75 minutes at 110 V, and nonspecific antibody binding was blocked for 1 hour in PBS with 0.1% Tween-20 and 5% nonfat milk at room temperature. Membranes were washed for 20 minutes in Tween/PBS and then incubated with the primary antibody of SERCA2a, PKA ,PP1 α and p-PLB (dilution 1:1000) at 4°C overnight. After washout of the primary antibody, membranes were incubated for 1 hour with anti-mouse IgG horseradish peroxidase-linked secondary antibody (dilution 1: 1000) and extensively washed again before exposing to an X-ray film for 2 min for chemiluminescent detection. Films were scanned into a computer, and band densities for protein were quantified with densitometric analysis by use of the UVIdoc Image program. Targeted bands were normalized to cardiac β -actin.[12]

Statistics

Data were expressed as means ± SE. We used ANOVA to compare data between groups. Statistical significance was accepted at the level of $P<0.05$.

Results

Effects of valsartan on cardiac structure and performance during the development of HF

Compared to control rabbits, the left ventricular end systolic and diastolic dimension, and inter-ventricular septal thickness in the HF rabbits without Valsartan were increased significantly (P<0.01, Table 1). Furthermore, in untreated failing rabbits, left ventricular end diastolic pressures were elevated, whereas the ejection fractions were decreased. These data indicated that ventricular remodeling was induced by chronic volume and pressure overload, and systolic function was impaired in the untreated failing group. But in Valsartan-treated HF rabbits with chronic volume and pressure overload, the cardiac systolic function was improved and left ventricular hypertrophy was prevented compared with the untreated rabbits.

Effects of valsartan on calcium uptake of SR

In this experiment, the reduced degree of calcium concentration out of the SR, which equals to the difference between the calcium concentration before and after adding ATP divided by that before adding ATP, represents the SR calcium uptake. After 6 weeks of volume and pressure overload, the SR Ca^{2+} uptake (Table 2,Fig.1), the activity and the amount of SR Ca^{2+}-ATPase (Table 2 and Figure 2 A) were decreased, and the decreases were smaller in the HF with Valsartan group than in the untreated HF group. Figure 2 D compares the levels of Ser16-phosphorylated phospholamban (p-PLB) among the SR vesicles. There was a significant decrease in the basal level of phosphorylated PLB in the untreated failing SR vesicles. In the Valsartan-treated SR vesicles, this level of phosphorylated PLB was restored back toward normal.

Discussion

In the present experiments, our major findings included: (1) ventricular remodeling and systolic dysfunction induced by chronic volume and pressure overload in the HF group. (2) Calcium reuptake of SR, activity of SERCA2a and PKA decreased in heart failing myocytes. (3) Down regulations of p-PLB, SERCA2a and PKA, but up regulation of PP1 α in ventricular samples from the failing rabbits. All of these changes were smaller in HF with Valsartan treatment.

During diastole, a reduction of $[Ca^{2+}]i$ is produced either by pumping back into the SR by the SERCA2a, to maintain the SR content for next beat, or outside of the cell largely by the Na^{+}-Ca^{2+} exchanger. Hobai and his colleagues have shown decreased SR Ca^{2+} content is responsible for defective systolic function in failing heart [2], but still conversely[4].

Table 1 Cardiac structure and performance data($\bar{x}$± SD)

	Controls (n=11)	HF (n=11)	HF with Valsartan (n=8)
VEDP (mm Hg)			
Before operation	-1.0 ± 0.9	-1.2 ± 0.8	-0.5 ± 1.1
6w operation	-0.5 ± 1.0	23.0 ± 2.3*	2.2 ± 0.7†
IVSth (mm)			
Before operation	1.9 ± 0.3	2.2 ± 0.3	2.2 ± 0.2
6w operation	2.1 ± 0.2	2.9 ± 0.5*	2.3 ± 0.5†
LVPWd(mm)			
Before operation	1.9 ± 0.2	2.2 ± 0.4	2.3 ± 0.2
6w operation	2.0 ± 0.2	2.9 ± 0.8*	2.1 ± 0.5†
LVEDD (mm)			
Before operation	14.5 ± 1.4	15.8 ± 2.4	15.7 ± 1.1
6w operation	13.3 ± 1.8	21.4 ± 2.5*	17.6 ± 1.9†
LVESD (mm)			
Before operation	8.7 ± 0. 8	10.1 ± 1.9	8.9 ± 0.8
6w operation	8.3 ± 1.1	17.2 ± 2.1*	11.6 ± 1.6†
EF (%)			
Before operation	74.2 ± 2.9	76.6 ± 2.9	74.7 ± 2.4
6w operation	71.9 ± 4.5	38.5 ± 6.0*	64.5 ± 3.6†

LVEDP, left ventricular end-dystolic pressure; IVSth, interventricle septal thickness; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; and EF, ejection fraction; *P<0.01 *vs* before operation and controls after 6-week operation; †P<0.05 *vs* HF after 6-week operation.

Table 2 Expressions and activities of calcium regulatory proteins($x \pm SD$)

	Controls (n=11)	HF (n=11)	HF with Valsartan (n=8)
Ca^{2+} uptake of SR (%)	95.52±2.12	54.39±7.87*	81.7±2.86†
SERCA2a			
Abundance	1.02± 0.02	0.69 ± 0.04*	0.85±0.02†
Activity (μmol/mg/h)	15.01±1.00	8.32±0.15*	12.46±1.00†
p-PLB abundance	0.44±0.17	0.23±0.28*	0.40±0.34†
PKA			
Abundance	1.05± 0.08	0.61±0.03*	0.83±0.09†
Activity (pmol/ μg/min)	1.85±0.05	1.09±0.09*	1.51±0.03†
PP1 α abundance	0.39± 0.07	1.25±0.06*	0.78±0.12†

***P<0.01 *vs* controls; †P<0.05 *vs* HF; abundance: ratio to cardiac β -actin.**

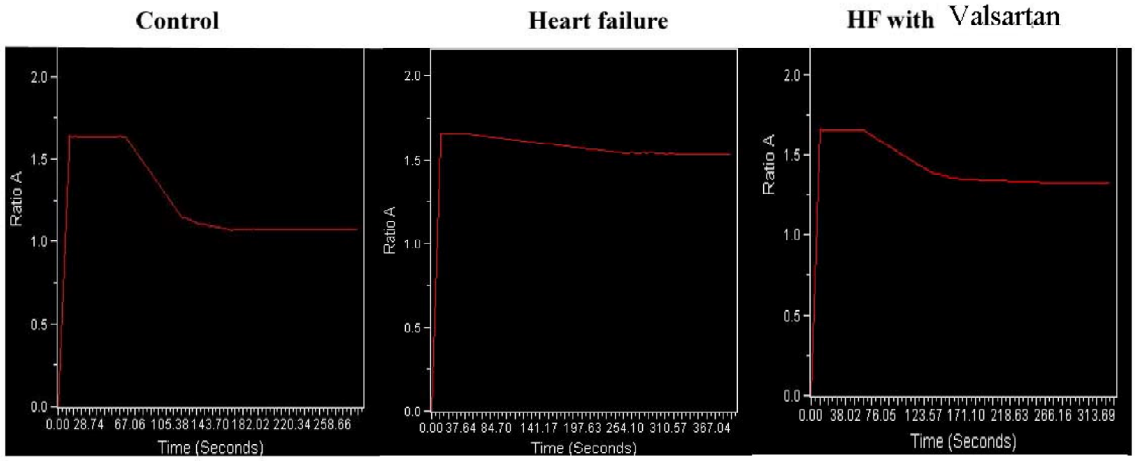

Fig.1 Typical curves of calcium reuptake of SR. The reduction degree of the R340/380 in HF was less than that in controls, but improved by valsartan(P<0.05).

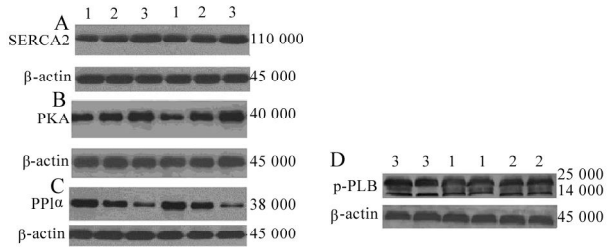

Fig .2 expression of calcium regulatory protein. 1,2,3 represent the HF, HF with Valsartan and controls respectively. The expression of SERCA2a,p-PLB, PKA was reduced whereas PPL α was increased in HF compared with the controls, but the aboundance of SERCA2A, p-PLB and PKA in HF with Valsartan was greater and PPL α was less than that in HF.

Therefore, we prepared the SR vesicles to take Ca^{2+} uptake assays directly and measure the activity of SERCA2a. We found that SR Ca^{2+} uptake was reduced in failing myocytes due to decreased SERCA2a activity compared with controls. These changes, consisted with other studies, would account for the reduction of SR Ca^{2+} storage that result in decreased SR Ca^{2+} release, which contribute to the systolic dysfunction. [3,13]

The activity of SERCA2a was regulated by its interaction with the inhibitory accessory protein-PLB. Phosphorylation of PLB relieves the inhibition of SERCA 2a, thereby stimulate its activity.[14] Our results indicated moderate decrease of both SERCA2a and ser16-p-PLB. Less abundance of p-PLB may be due to decreased activity and abundance of PKA and increased abundance of PP1 α , which phosphorylated and dephosphorylaed PLB respectively.[14, 15] These changes account for decreased SERCA2a activity,[16, 17] which was attributed to reduced SR Ca^{2+} uptake.[13]

Chronic treatment with the angiotension -receptor blocker Valsartan in chronic heart failure could improve the cardiac function and prognosis.[8] Our study indicated Valsartan prevent the development of left ventricular remodeling and cardiac systolic dysfunction induced by chronic volume and pressure overload. This research also showed that Valsartan could elevate the activity and abundances of PKA and decreased the expression of PP1 α ,[18] resulting in increased abundance of ser16-p-PLB, which may

be attributed to increment of the SERCA2a activity and SR calcium uptake. These results agreed to other studies.[19,20] Ju et al[21] suggested that Ang Ⅱ could induce decreased activity and expression of SERCA2a, and Shao et al[22] found that imidapril could modified these changes. Therefore, we consider that renion-angiotension-system may participate in the alteration of calcium regulatory proteins in heart failure. Through blocking the angiotension receptor, Valsartan ameliorates the changes of SERCA2a and its regulatory proteins, resulting in preventing the development of heart failure.

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