# Dispersion of ventricular mRNA of RyR2 and SERCA2 associated with arrhythmogenesis in rats[1]

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## ABSTRACT

**AIM:** To investigate the effect of CPU86017 on the changes of mRNA abundance of different calcium handling system in infarcted heart. **METHODS:** Rats were subjected to left coronary ligation to induce myocardial infarction (MI). The treatment with either propranolol (Pro) 5 mg/kg ip or CPU86017 1, 2, and 4 mg/kg ip was initiated on the next day of operation and continued for 20 d. Medication with isoproterenol (Isop) 3 mg/kg sc started on the d 17-21. Ventricular mRNA abundance of ryanodine receptor 2 (RyR2), sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA2), L-type $Ca^{2+}$ channel, and $Na^{+}/Ca^{2+}$exchanger (NCX1) were measured. **RESULTS:** Arrhythmic scores (AS) in the Isop group was raised up to 5.27±1.75 ($P$<0.01) *vs* myocardial infarction group 2.25±2.04 and sham group 1.50±1.73. The AS was depressed by Pro (1.63±1.53, $P$<0.01 *vs* Isop), and CPU86017 2 and 4 mg/kg (3.00±1.24, and 1.70±1.85, $P$<0.01 *vs* Isop). The significant dispersion of depressed mRNA abundance of RyR2 and SERCA2 was associated with an increase in AS in Isop group, and it was much depressed in the left than the right ventricle. The dispersion and depression of mRNA were restored significantly by Pro and CPU86017, associated with suppression on AS. In Isop group, the mRNA abundance of L-type $Ca^{2+}$ channel was not changed; and a moderate increase in the mRNA of NCX1 was seen, the changes were regressed by Pro and CPU86017. **CONCLUSION:** Isop-induced arrhythmogenesis in MI heart was correlated mainly with a dispersion of depressed mRNA abundance in ventricle likely due to the consequence of PKA over-phosphorylation. A suppression of arrhythmia by Pro and CPU86017 resulted from a regression of the dispersion and depression of RyR2 and SERCA2.

## INTRODUCTION

Severe ventricular arrhythmias are remained great risk of mortality in patients with cardiovascular disorders and cover about 50 % of the death[1,2]. The progression of heart failure is developed by abnormality of the calcium handling system in myocardium[3,4]. However, it is still uncertain whether the abnormality is involved in the pathological derangement of enhancing severity of cardiac arrhythmias. Phosphorylated sarcoplasmic reticulum (SR) ryanodine receptor 2 (RyR2) and SR $Ca^{2+}$- ATPase (SERCA2) are activated to release or up-take calcium from the SR. On the other hand, in case of a failing heart an over-phosphorylation of the two has been focused and is attributed to an access of PKA activated by the pathological process of cardiac failure[5-7]. On the stimulation of β-adrenergic receptors (βARs) by exposure to isoproterenol (Isop), it is

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interesting to observe and compare whether arrhythmogenesis is induced and what is the most prominent change of the mRNA abundance among RyR2, SERCA2, L-type $Ca^{2+}$ channel, and $Na^+/Ca^{2+}$exchanger (NCX1) in an infarcted heart. Since the dispersion of the function among ion channels of $I_{Kr}$, $I_{Ks}$, $I_{KI}$, $I_{Ca.L}$, and $I_{to}$ has been found in remodeled heart by L-thyroxin, and this shows a tendency of exacerbated arrhythmias on ischemia/reperfusion[3,8-10], so it is reasonable to consider that exaggerated cardiac arrhythmia can be the result of the dispersion of the intracellular ion channels on the SR.

The ion channelopathy contributing to arrhythmogenesis could be considered in two different categories, the basic and activated ion channelopathy[1,9]. The basic phase of ion channelopathy in an infarcted myocardium has no tendency to enhance cardiac arrhythmias on episode of ischemia/reperfusion. A more serious ion channelopathy of an affected myocardium shows a tendency of enhancing cardiac arrhythmias after ischemia/reperfusion of coronary artery and a disordered intracellular calcium handling system is involved. It is evidenced in L-thyroxin induced remodeling that multichannels are disordered in the lipid membrane in association of abnormal calcium dealing system in an affected heart[3,10-12]. Therefore, we intended to investigate whether there existed dispersion of mRNA abundance of different intracellular calcium dealing system, and effects of propranolol (Pro) and CPU86017.

## MATERIALS AND METHODS

**Drugs** Isop (Shanghai Harvest Pharmaceutics Co, Ltd); Pro (Sigma); CPU 86017 (purity>99 %; Department of Pharmaceutical Chemistry, China Pharmaceutical University)

**Study protocol** Myocardial infarction (MI) was induced by permanent ligation of the left anterior coronary artery using a standardized surgical procedure. Male Sprague-Dawley rats (203±16 g; Center of Experimental Animals, China Pharmaceutical University) were anesthetized with diethylether. Then rats were artificially ventilated with room air. Electrocardiogran (ECG) was monitored. The heart was exposed, and a 5-0 silk suture was placed around the left coronary artery (2-3 mm from its origin), then the suture was tied securely. In rats that underwent sham surgery, suture was through underneath the coronary artery without ligation.

Twenty-four hours after surgery , the survival rats were divided randomly into 6 groups: (1) MI; (2) Isop (MI rats by administration of 3 mg/kg Isop sc on d 17-21 after MI); (3) Pro (3 mg/kg Isop sc on d 17-21 after MI; Pro treatment started at 24 h after MI, ip, 5 mg/kg for 20 d); (4)-(6)CPU 86017 1, 2 , and 4 mg/kg (3 mg/kg Isop sc on d 17-21 after MI; CPU 86017 treatment started at 24 h after MI, ip, for 20 d). Sham surgical rats were served as sham group.

**Ischemia-reperfusion arrhythmia** The rats were anesthetized with pentobarbitone (50 mg/kg, ip) and heparin (1000 U/kg, ip). The heart was excised rapidly and perfused via the aorta in the Langendorff mode with Tyrode's solution (37 ºC) saturated with 95 % $O_2$ and 5 % $CO_2$. Perfusion pressure was maintained at 47.8 mmHg. Three 0.1-mm silver electrodes were placed in the left ventricle, right atrium, and aorta to record ECG throughout the experimental period. The heart was initially given a 15-min aerobic perfusion; ischemia was produced by reducing the perfusion flow to one tenth for 10 min; then the perfusion flow was resumed for 20 min (reperfusion). Arrhythmia score (AS) was evaluated according to previous study[13].

**RNA preparation** Total RNA was isolated from 100 mg frozen right and left ventricular muscle tissue by using 1 mL Trizol (BBI). The amount of total RNA was determined spectrophtometrically (Gene Ltd) at a wavelength of 260 nm, and the RNA was stored at -80 ºC for reverse transcription-polymerase chain reaction (RT-PCR).

**Semi-quantitative RT-PCR** A quantity of 0.4 μg RNA was reacted in a 25 μL mixture containing 1×reaction buffer, $MgSO_4$ 1 mmol/L, 200 μmol/L of each dNTP, specific primer 1 μmol/L, 2.5 U AMV reverse transcriptase, and 2.5 U TfI DNA polymerase. This mixture was overlaid with 20 μL mineral oil. After an incubation for 45 min at 45 ºC to initiate the synthesis of cDNAs, the reverse transcription was inactivated at 94 ºC for 2 min. PCR was performed with the following conditions: denaturing at 94 ºC for 30 s, annealing at 60 ºC for 1 min, extension at 68 ºC for 2 min, 40 cycling, and final extension at 68 ºC for 7 min (BIO-RAD Gene Cycler™). The specific primers were as follows (Sangon): RyR2 sense 5´-GAATCAGTGAGTTACTGGGCATGG-3´ and antisense 5´-CTGGTCTCTGAGTTCTCCAAAAGC-3´ (635 bp)[5]; SERCA2 sense 5´-ATGAGATCACAGCTA-TGACTGGTG-3´ and antisense 5´-GACTTGCACA-TCTCTATGGTGACTAG-3´ (638 bp)[14]; NCX1 sense TCTTCAGAAGTCTCGGAAGAT

and antisense CACTTCCAGCTTGGTGTGT (644 bp)[15]; L-type $Ca^{2+}$ channel sense 5′-ATCCCCAAGAACCA-GCACCAG-3′ and antisense 5′-GGTGATGGAGATGC-GGGAGTT-3′ (372 bp)[16]. To ensure a fixed amount of initial mRNA paralleled β-actin amplification was performed using the following oligonucleotides: sense 5′-GGTATGGGTCA-GAAGGACTCC-3′ and antisense 5′-TGATCTTCA-TGGTGCTGCTAGGAGCC-3′ (780 bp).

The amplification products were separated by agarose gel electrophoresis (1.5 %), stained with ethidium-bromide, and visualized under ultraviolet, and scanned by Magemaster VDS (Pharmacia Biotech). The density of the bands was analyzed by computer with Laboratory Work 4.0 (Protein scan systems, Gene Ltd). The relative density of bands for each mRNA was divided by the band for the internal control β-actin. Sham and MI groups: $n$=8; Isop, Pro, and CPU 86017 groups: $n$=9.

**Statistical analysis** All data were presented as mean±SD. Difference between groups were evaluated by analysis of variance (ANOVA) followed by $t$-test. Statistical significance was accepted at level of $P$<0.05.

## RESULTS

**Survival rate of rats and weight index of the heart** There was no death in the sham-operated rats. Three weeks after surgery, the survival rate was calculated for each group (Tab 1). In Isop group, the survival rate decreased markedly. The survival rate was increased after treatment with Pro and CPU86017 (2 and 4 mg/kg). In MI and Isop group, heart weight index, right ventricle (RV) weight index, and left ventricle (LV) weight index were increased significantly. It indicated that ventricular hypertrophy occurred. Pro and CPU86017 (4 mg/kg) could regress the hypertrophy induced by MI and Isop (Tab 2).

**Ischemia-reperfusion arrhythmia** Compared with sham group, there was no significant difference in MI group. In Isop group, AS increased markedly. Pro and CPU86017 (2 and 4 mg/kg) improved the arrhythmia (Tab 3).

**Change of mRNA expression of $Ca^{2+}$ handling system** There was no significant difference in mRNA expression of $Ca^{2+}$ handling system in MI group compared with that of sham group. In Isop group, mRNA expression changed markedly. The mRNA abundance of RyR2 and SERCA2 were decreased by 83.6 % and 77.6 % respectively in LV, and decreased by 36.7 % and 38.1 % in RV, respectively ($P$<0.01 *vs* MI). But

**Tab 1. The survival rate for each group.**

| Groups | Survival number | Survival rate/% |
|---|---|---|
| Sham | 10/10 | 100 |
| MI | 12/13 | 92.3 |
| Isop | 9/17 | 52.9 |
| Pro | 11/13 | 84.6 |
| CPU86017/mg·kg$^{-1}$ | | |
| 1 | 8/15 | 53.3 |
| 2 | 10/15 | 66.7 |
| 4 | 10/13 | 76.9 |

**Tab 2. Comparison among heart weight index (HWI), right ventricle weight index (RWI), and left ventricle weight index (LWI, myocardial weight dividing by body weight). Mean±SD. $^{c}P$<0.01 *vs* sham group. $^{e}P$<0.05, $^{f}P$<0.01 *vs* Isop group.**

| Groups | Weight index/mg·g$^{-1}$ | | |
|---|---|---|---|
| | HWI | RWI | LWI |
| Sham ($n$=10) | 3.82±0.41 | 0.61±0.10 | 2.95±0.35 |
| MI ($n$=12) | 4.65±0.57$^{c}$ | 1.19±0.36$^{c}$ | 3.25±0.29$^{f}$ |
| Isop ($n$=9) | 5.04±0.46$^{c}$ | 1.16±0.09$^{c}$ | 3.78±0.46$^{c}$ |
| Pro ($n$=11) | 4.43±0.23$^{f}$ | 0.88±0.25$^{f}$ | 3.31±0.14$^{f}$ |
| CPU86017/mg·kg$^{-1}$ | | | |
| 1 ($n$=8) | 5.06±0.62 | 1.20±0.45 | 3.80±0.52 |
| 2 ($n$=10) | 5.02±0.17 | 1.12±0.14 | 3.88±0.18 |
| 4 ($n$=10) | 4.38±0.22$^{f}$ | 0.91±0.18$^{f}$ | 3.29±0.32$^{e}$ |

**Tab 3. Score of arrhythmia induced by an ischemia-reperfusion in isolated rat hearts. Mean±SD. $^{c}P$<0.01 *vs* sham group. $^{f}P$<0.01 *vs* Isop group.**

| Groups | Score |
|---|---|
| Sham ($n$=10) | 1.50±1.73 |
| MI ($n$=12) | 2.25±2.04$^{f}$ |
| Isop ($n$=9) | 5.27±1.75$^{c}$ |
| Pro ($n$=11) | 1.63±1.53$^{f}$ |
| CPU86017/mg·kg$^{-1}$ | |
| 1 ($n$=8) | 5.30±1.65 |
| 2 ($n$=10) | 3.00±1.24$^{f}$ |
| 4 ($n$=10) | 1.70±1.85$^{f}$ |

the mRNA abundance of NCX1 were increased by 63.8 % in LV, and 40.4 % in RV ($P$<0.01 *vs* MI). There was significant difference in mRNA expression of RyR2 and SERCA2 between LV and RV in Isop group ($P$< 0.01), and it was more depressed in the LV against the RV indicating dispersion between two sides of ventricle. The mRNA abundance of L-type $Ca^{2+}$ channel had no significant difference. After the treatment with Pro and CPU86017 (2 and 4 mg/kg), the abundance of mRNA was normalized and the difference between LV and RV disappeared (Fig 1-4).

## DISCUSSION

In post-MI rats continuously received Isop resulted in a β ARs overactivation to mimic the hypersympathetic overactivity which was likely related to develop of ion channelopathy and heart failure[17-20]. The AS after hypoxia/reperfusion was not increased in the MI group, however, it was raised after medication with Isop as-

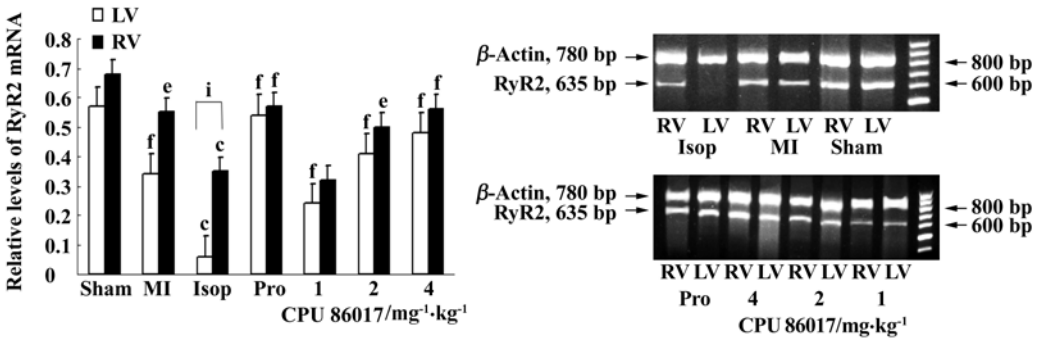


**Fig 1. The mRNA expression levels of RyR2 by RT-PCR. $n$=9-12. Mean±SD. $^{c}P$<0.01 *vs* sham group. $^{e}P$<0.05, $^{f}P$<0.01 *vs* Isop group. $^{i}P$<0.01 compared between LV and RV.**

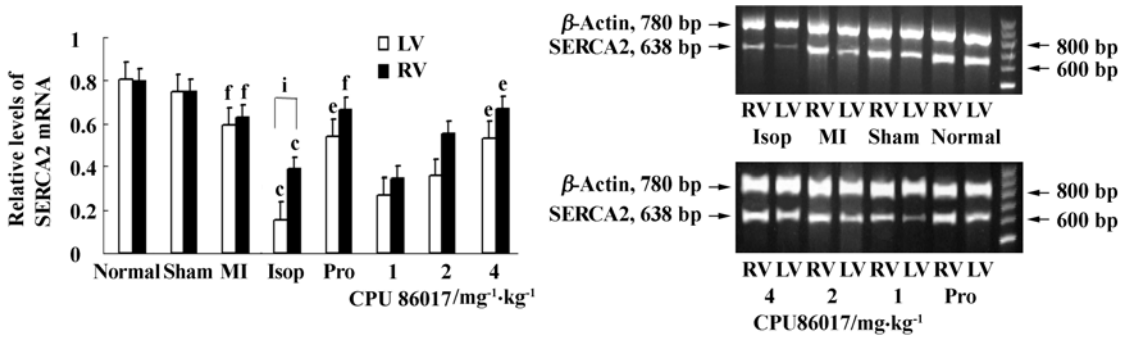


**Fig 2. The mRNA expression levels of SERCA2 by RT-PCR. $n$=9-12. Mean±SD. $^{c}P$<0.01 *vs* sham group. $^{e}P$<0.05, $^{f}P$<0.01 *vs* Isop group. $^{i}P$<0.01 compared between LV and RV.**

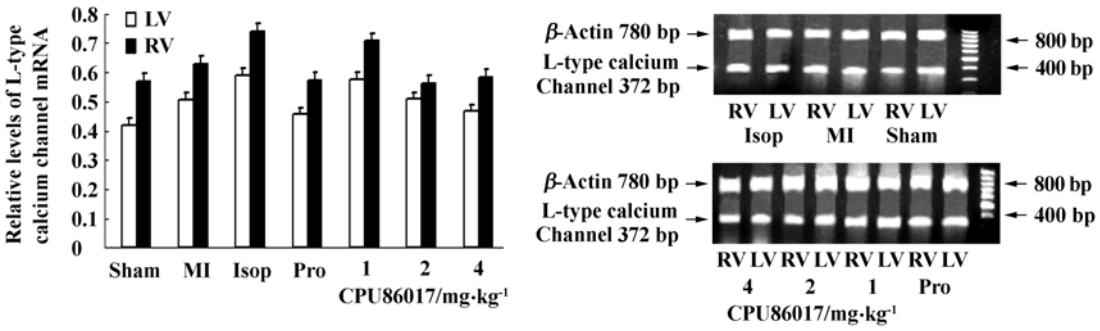


**Fig 3. The mRNA expression levels of L-type calcium channel by RT-PCR. Mean±SD.**

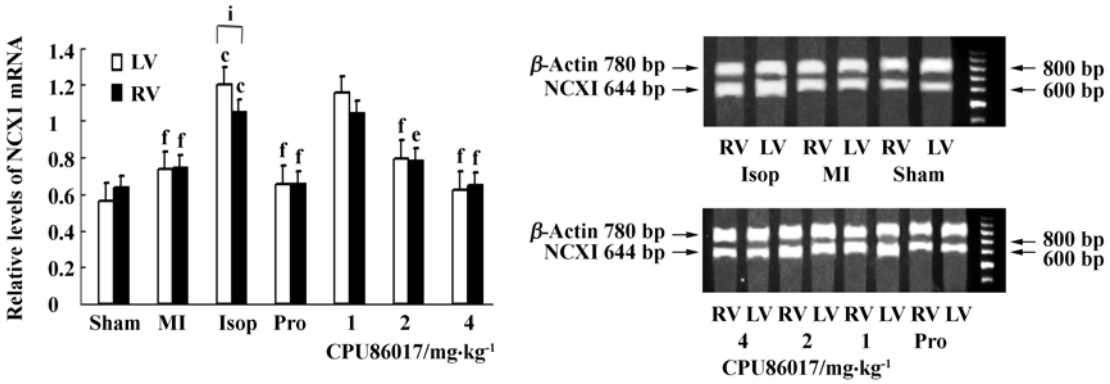


**Fig 4. The mRNA expression levels of NCX1 by RT-PCR. Mean±SD. $^{c}P<0.01$ *vs* sham group. $^{e}P<0.05$, $^{f}P<0.01$ *vs* Isop group. $^{i}P<0.01$ compared between LV and RV.**

sociated with a significant ventricular hypertrophy. It was implied that an involvement of β ARs activation was prominent to promote the appearance of exaggerated cardiac arrhythmias. We observed that Pro was effective to suppress the arrhythmias markedly together with a reduction in the myocardial hypertrophy. CPU 86017, which is derived from tetrahydroberberine not only had mild β-blockade, potent α-blockade effects[21], but also had blockade on multi-channels including the L-type $Ca^{2+}$ channel[22]. The severity of cardiac arrhythmias developed by hypoxia/reperfusion was exaggerated significantly by an addition of a β-agonist and an α-agonist[23]. A blockade on the α-receptor was likely to play a role in the antiarrhythmic mechanism of CPU86017. The medication of Isop created a series of oxygen free radicals by the oxidation of the catecholamine, and oxidative stress was reconsidered as a primary factor in morbidity of cardiac failure. The antioxidative effect of CPU86017 and Pro[24] was well impressed, so it contributed to reduce the incidence of cardiac arrhythmias on ischemia/reperfusion episode.

The depressed mRNA abundance of RyR2 and SERCA2 was evident and these were coincided in hypertrophied myocardium[5,7]. A phenomena of "leaky" caused an increased $Ca^{2+}$ during the diastolic phase but a reduced $Ca^{2+}$ release in the systolic phase[25]. It was very interesting to find that the reduction in mRNA abundance of the RyR2 and SERCA2 in the right and left ventricle was different significantly, with the more depressed RyR2 and SERCA2 on the left side. This phenomenon of dispersion indicated that the imbalance of $Ca^{2+}$ ion in the cytosol of myocytes was more serious in the LV; therefore, it offered a tendency of arrhythmogenesis and enhancement of arrhythmias on hypoxia/reperfusion episode, and it was likely to be equal important with the dispersion of the ion channels in membrane[3,8-10].

The over-phosphorylation by PKA was considered as an important factor causing a depression of RyR2 and SERCA2, so, it might be deduced that the extent of the phosphorylation on the two ventricles was different; an over-dispersed-phosphorylation of the calcium handling system existed in the MI heart stimulated by Isop, this offered a status prone to the occurrence of severe cardiac arrhythmias. Intervention of Pro and CPU86017 resulted in a suppression of arrhythmia associated with a recovery of the dispersion of RyR2 and SERCA2 in the two ventricles, particular in the improvement on the left side.

There were likely two different types of phosphorylation of RyR2 and SERCA2 by PKA. The up-regulation of the RyR2 and SERCA2 was possibly characterized by an increment of calcium released intracellularly in the cardiac remodeling by L-thyroxin[12]. When an infarcted heart was medicated with Isop, RyR2 was hyperphosphorylated by PKA in failure hearts[4].

The L-type $Ca^{2+}$ channel was not involved in the development of the cardiac arrhythmias in this model and the NCX1 might play a minor role in this respect. The selectivity of the impairment of the calcium handling system in an infarcted heart delivered information properly that the RyR2 and SERCA2 were the most important elements in the pathogenesis of cardiac arrhythmias and cardiac dysfunction.

The dispersion of the intracellular calcium channels among the RyR2, SERCA2, and NCX1 served as a basis of arrhythmogenesis. By the intervention with Pro and CPU86017, the dispersion was recovered in a dose-response manner and associated with improvement of hypertrophy and arrhythmias.

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