Original Article

Isoproterenol-induced FKBP12.6/12 downregulation is modulated by ET_A and ET_B receptors and reversed by argirhein, a derivative of rhein

Guo-lin ZHANG¹, De-zai DAI^{1,*}, Tao XI^{1,*}, Xiao-dong CONG², Yun ZHANG², Yin DAI¹

¹China Pharmaceutical University, Nanjing 210009, China; ²Zhejiang Chinese Medical University, Hangzhou 311401, China

Aim: To investigate which endothelin receptors mediated isoproterenol (ISO)-induced downregulation of FKBP12.6/12 in cardiomyocytes and study whether argirhein, a novel compound containing rhein and L-arginine that has anti-inflammatory activity, could reverse the downregulation of FKBP12.6/12 induced by ISO.

Methods: Neonatal rat cardiomyocytes were incubated with ISO to downregulate FKBP12.6/12. Then the cells were treated with a selective ET_A blocker (PD156707) and a ET_B blocker (IRL1038), a dual ET_A/ET_B antagonist (CPU0213), and argirhein, respectively. FKBP12.6/12 expression was assayed by RT-PCR, Western blot, and immunocytochemistry.

Results: The expression of FKBP12.6 mRNA was reduced by 37.7% ($P<0.01$) and 28.9% ($P<0.05$) relative to the control by ISO 1 and 0.1 $\mu\text{mol/L}$, respectively, but no response to ISO 0.01 $\mu\text{mol/L}$ was observed *in vitro*. FKBP12.6/12 protein expression was reduced by 47.2% ($P<0.01$) and 37.8% ($P<0.05$) by ISO 1 and 0.1 $\mu\text{mol/L}$, respectively. This decrease was reversed significantly by PD156707, or IRL1038, and CPU0213. CPU0213 was more potent than either PD156707 or IRL1038. Argirhein 10 $\mu\text{mol/L}$ blunted the downregulation of FKBP12.6/12 by ISO, as demonstrated by the rising mRNA and protein levels and by the fluorescent density of the ISO-incubated cardiomyocytes.

Conclusion: In cardiomyocytes, the ISO induced downregulation of FKBP12.6/12 is modulated by both ET_A and ET_B. A new compound, argirhein, reversed the down-regulation of FKBP12.6/12 expression in myocardial cells stimulated with ISO.

Keywords: isoproterenol; FKBP12.6/12; ET_A receptor; ET_B receptor; argirhein; rhein

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Introduction

Severe arrhythmias and congestive heart failure have become major problems affecting humans, and the annual incidence of sudden cardiac death (SCD) in the general population is estimated to be 1 in 1000^[1]. Mutations in the potassium channels KCNQ2 and KCNH2, and in the sodium channel SCN5A serve as markers only for polymorphism, and severe arrhythmias may occur in the presence of triggering factors^[2]. Trigger factors, including stress caused by β -adrenergic stimulation and some medications, affect K⁺ currents during repolarization, resulting in tachyarrhythmias in patients with mutated genes. Tricyclic antidepressants may trigger the appearance of Brugada syndrome (Brugada ECG and life-threatening ventricular arrhythmias) in patients with the SCN5A polymorphism (His558Arg)^[3]. Mutation of the ryanodine receptor type 2 (RyR2) alone does not appear to cause tachyarrhythmias;

however, patients with RyR2 mutations may present with CPVT (catecholaminergic polymorphic ventricular tachyarrhythmias) while engaging in physical exercise, which is always associated with β -adrenoceptor stimulation^[4]. Life threatening arrhythmias are likely to occur in patients with cardiac disease such as heart failure, in which multiple steps are needed to trigger events, including profound activation of β -adrenoceptor^[5, 6], and many causal factors are implicated in the pathogenesis of failing hearts in which an excess of reactive oxygen species (ROS) and inflammatory factors exist, resulting from the downstream events of β -adrenergic stimulation. Some inflammatory factors that have been revealed in the pathogenesis of congestive heart failure may participate in mechanisms underlying life threatening arrhythmias in failing hearts^[7].

FKBP12.6 (calstabin 2) is a key subunit that binds to RyR2 at the sarcoplasmic reticulum (SR) and is involved in calcium handling activity in the myocardium. FKBP12.6 also plays a role in the molecular mechanisms underlying severe arrhythmias and cardiac insufficiency^[8]. CPVT may be the conse-

* To whom correspondence should be addressed.

E-mail dezaidai@vip.sina.com; xi_tao18@sina.com; azai308@163.com

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quence of dissociated/downregulated FKBP12.6/12 related to profound β -adrenoceptor activation that keeps RyR2 channels closed loosely, allowing calcium leaks during diastole. RyR2 is phosphorylated by protein kinase A (PKA)^[9]. FKBP12.6/12 dissociation from the binding site at RyR2 is likely due to a decrease in affinity, but this hypothesis is still controversial^[10]. Elevated free calcium levels during diastole contribute to an increased risk for both cardiac tachyarrhythmias and exacerbated cardiac failure. Downregulation of FKBP12.6/12 in cardiomyocytes can be reproduced by isoproterenol (ISO) and can be induced by either endothelin-1 (ET) or H_2O_2 ^[11]. The resulting calcium leaks can be predicted by the downregulation of FKBP12.6/12^[12]; therefore, downregulated FKBP12.6/12 may be taken as a surrogate for calcium leaks, indicating an increased risk for severe arrhythmias and the deterioration of cardiac performance^[13].

ET-1 is a cytokine that actively participates in inflammatory reactions and ROS genesis through activating NADPH oxidase^[14]. An activation of the ET system is always associated with NADPH oxidase, forming the ET-NADPH oxidase pathway that is implicated in many cardiovascular diseases^[15]. The biological activities of ET-1 are the result of the stimulation of ET receptors A (ET_A) and B (ET_B). In our previous study, ET-1 was shown to cause the downregulation/dissociation of FKBP12.6/12 in cardiomyocytes^[11]; however, it is unclear whether ET_A and ET_B play separate roles in this process.

Argirhein, a new synthetic compound, contains rhein linked to *L*-arginine with a hydrogen bond in its moiety (Figure 1). Rhein relieves liver fibrosis and injury through anti-inflammatory activity^[16, 17]. When argirhein is used as a medication, rhein can be released from argirhein and then display its anti-inflammatory activity, which may protect cardiomyocytes from ISO-induced insults; therefore, the activity of argirhein could be relevant in attenuating the downregulation of FKBP12.6/12 at the SR. We hypothesized that the downregulation of FKBP12.6/12 by ISO causes a disturbance in calcium homeostasis, which worsens arrhythmogenesis and cardiac performance. This downregulation could be mediated by ET_A and ET_B individually. Given the anti-inflammatory activity of rhein, argirhein may have an activity similar to that of ET blockers, which play a role in protecting the myocardium by alleviating ISO-induced downregulation of FKBP12.6/12.

Figure 1. Chemical structure of argirhein.

Materials and methods

Animals

Animal handling procedures were conducted in accordance with the Laboratory Animal Regulations of the Bureau of Science and Technology, Jiangsu Province, China.

Reagents

Isoproterenol was purchased from Shanghai Hefeng Medicine Company (Shanghai, China). PD156707, a selective endothelin receptor A antagonist, and IRL-1038, a selective endothelin B receptor antagonist, were purchased from Sigma and Gen-script Corporation, USA, respectively. M-MLV (Promega, USA) and Taq DNA Polymerase (Tiangne) were purchased from Nanjing Tianwei Corp, China. Polyclonal goat anti-FKBP12.6/12 IgG was obtained from Santa Cruz Biotechnology Inc, USA. Polyclonal rabbit anti-actin-IgG and FITC-conjugated rabbit anti-goat IgG were purchased from Boster, Wuhan, China. HRP-conjugated rabbit anti-goat IgG was from Dako, USA. Argirhein (AR) was sourced from Zhejiang Chinese Medical University.

Cell culture

Neonatal Sprague-Dawley rats were obtained from the Experimental Animal Center of Nanjing. Neonatal ventricular myocytes were obtained and cultured as described previously^[9]. Briefly, myocytes were obtained and cultured in 20% FBS-DMEM culture medium with BrdU to suppress the growth of fibroblasts. The culture was changed to serum-free DMEM medium after three days, at which time the myocytes reached confluence. Except for the control groups, myocytes were incubated with ISO (0.01, 0.1, or 1 $\mu\text{mol/L}$) for 18 h to determine the appropriate ISO concentration. Cells were incubated with PD156707, IRL-1038, CPU0213 and argirhein (AR) at the 3 doses to prevent the adverse effects of ISO on FKBP12.6/12 in cardiomyocytes.

Semi-quantitative RT-PCR

After an 18-h incubation, RNA was extracted with Trizol solution, and cDNA was synthesized as described previously^[12]. RT-PCR was performed in a volume of 25 μL , and the products were detected in 2% agarose. The target gene was quantified using β -actin as an internal control. The sequences of the forward and reverse primers for FKBP12.6 (length 427 bp) and β -actin (580 bp) are listed below: 5'-AAGGAAGGACG-GAAGTG-3' and 5'-GAATAGAAGACAACCGACG-3'; and 5'-GCCTCAAGATCATCAGCAAT-3' and 5'-AGGTCCACCA-CTGACACCTT-3', respectively. The densities of the bands were analyzed using a gel imaging analysis system (GeneGenius, Syngene, England), and the relative density of each DNA band was obtained by dividing by the density of β -actin.

Western blotting

Protein was extracted from the incubated myocytes as described previously^[18]. Briefly, after determination of the pro-

tein concentration, the supernatant was stored at -20 °C before use. Aliquots of samples were heated to 98 °C in a loading buffer and fractionated using 10% SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane that was blocked with nonfat milk (5% *w/v*). The blocked nitrocellulose membranes were incubated at 4 °C overnight with the specific primary antibody. After 3 washes, the blots were incubated with horseradish peroxidase (HRP)-conjugated goat secondary antibody IgG for 1 h at room temperature. Antigen was detected with a DAB kit, visualized by imaging acquisition and quantified by densitometry. The relative abundance was obtained by normalizing the density of the FKBP12.6/12 protein against that of β -actin.

Immunocytochemical analysis

Cardiomyocytes were fixed with cold acetone for 10 min after incubation with ISO for 18 h. After the cells were dried at room temperature, the cell membrane permeability was increased by incubation with 1% Triton X-100 for 1 h. Then, cardiomyocytes were sealed with 2% bovine serum albumin for 1 h and incubated overnight with polyclonal goat anti-FKBP12.6/12-IgG. After being washed 3 times with PBS, the cardiomyocytes were incubated with FITC-conjugated rabbit anti-goat IgG for 1 h. Finally, cardiomyocytes were imaged by fluorescence microscopy and the gray density was assayed and compared^[19].

Statistical analysis

All data are presented as the mean $\pm$ SD and were analyzed with SPSS version 11.5 (USA). For statistical evaluation, a one-way analysis of variance was used, following Dunnett's test. The Student Newman Keuls test was performed when the variances were equal, and the Games-Howell test was used when the variances were not equal. A probability value of $P < 0.05$ was considered statistically significant.

Results

Downregulation of FKBP12.6/12

The downregulation of FKBP12.6/12 in cardiomyocytes responded to ISO at 3 doses *in vitro*. The expression of FKBP12.6 mRNA was reduced by 37.7% ($P < 0.01$) and 28.9% ($P < 0.05$) relative to the control at ISO concentrations of 1 $\mu\text{mol/L}$ and 0.1 $\mu\text{mol/L}$, respectively, but no response to ISO 0.01 $\mu\text{mol/L}$ was observed. A reduction in FKBP12.6/12 protein expression of the same magnitude was found by Western blotting; the reductions were 47.2% ($P < 0.01$) and 37.8% ($P < 0.05$) for 1 $\mu\text{mol/L}$ and 0.1 $\mu\text{mol/L}$, respectively (Figure 2A, 2B). Based on these results, an ISO concentration of 1 $\mu\text{mol/L}$ was adopted for further experiments.

Responses to ET antagonists

The expression levels of FKBP12.6/12 mRNA and protein were downregulated in the presence of ISO, and then selective blockade of either ET_A or ET_B was analyzed. The ET_A antagonist PD156707 suppressed the changes in protein abundance significantly at concentrations of 0.1 and 1 $\mu\text{mol/L}$ relative to

Figure 2. The mRNA (A) and protein (B) expression of FKBP12.6/12 in neonatal rat cardiomyocytes were downregulated after incubation with isoproterenol (ISO) for 18 h. $n=6$. Mean $\pm$ SD, ^b $P < 0.05$, ^c $P < 0.01$ vs control. The relative expression of mRNA and protein was normalized by β -actin.

ISO alone (Figure 3A, 3B). It was found that the ET_B antagonist IRL-1038 at a concentration of 1 $\mu\text{mol/L}$ only induced a reduction in the downregulation of FKBP12.6/12 toward the normal level (Figure 3D). At 0.1 $\mu\text{mol/L}$, PD156707 was able to upregulate the abundance of FKBP12.6/12 remarkably; however, IRL1038 had no significant effect. Thus, ET_A was more potent than ET_B in modulating changes in FKBP12.6/12 induced by ISO. mRNA and protein expression for FKBP12.6/12 were not changed in the absence of ISO after an 18-h incubation with either PD156707 or IRL-1038 alone (Figure 3E, 3F).

CPU0213, a dual ET_A and ET_B receptor antagonist, was added at concentrations ranging from 0.01 to 1 $\mu\text{mol/L}$ to test its effects on the changes in FKBP12.6/12 expression induced by ISO. CPU0213 was able to significantly reverse the downregulation of the levels of mRNA and protein of FKBP12.6/12 at all three ISO concentrations. At a concentration as low as 0.01 $\mu\text{mol/L}$, CPU0213 was able to elevate the depressed FKBP12.6/12 levels significantly, while no response was observed to either PD156707 or IRL-1038. It appears that a combined blockade of the two subtypes of ET receptors is more potent than a single selective blockade (Figure 4).

Argirhein upregulates FKBP12.6/12

Compared with the ISO group, the argirhein group showed a significant reversal of the ISO-induced downregulation of

Figure 3. Selective ET_A antagonist PD156707 (A, B) and ET_B antagonist IRL1038 (C, D) reversed the ISO-induced downregulated mRNA and protein expression of FKBP12.6/12 normalized by β -actin in neonatal rat cardiomyocytes, but the expression of FKBP12.6/12 was not affected when incubation with drugs PD156707 and IRL1038 in the absence of ISO (E, F). $n=6$. Mean $\pm$ SD. $^{\circ}P<0.01$ vs control. $^{\circ}P<0.05$, $^{\dagger}P<0.01$ vs ISO.

Figure 4. Dual ET antagonist CPU0213 reversed the ISO-induced downregulation of mRNA (A) and protein (B) expression of FKBP12.6/12 normalized by β -actin in neonatal rat cardiomyocytes. $n=6$. Mean $\pm$ SD. $^{\circ}P<0.01$ vs control. $^{\circ}P<0.05$, $^{\dagger}P<0.01$ vs ISO.

FKBP12.6/12 when argirhein was used at 10 μ mol/L (Figure 5A, 5B). The activity of argirhein was similar to those of the ET antagonists and was less effective than either CPU0213 or PD156707. Cardiomyocytes incubated with argirhein alone has not altered expression of FKBP12.6/12 mRNA and protein

(Figure 5C). This result indicates that argirhein counteracts the adverse effects of ISO on FKBP12.6/12 in cardiomyocytes.

Immunocytochemistry of FKBP12.6/12

In an immunocytochemistry assay, the fluorescence inten-

Figure 5. Downregulated expression of FKBP12.6/12 mRNA (A) and protein (B) normalized by β -actin were reversed by argirhein, but the expression of FKBP12.6/12 were not affected when incubation with argirhein in the absence of ISO (C). $n=6$. Mean $\pm$ SD. $^cP<0.01$ vs control. $^eP<0.05$ vs ISO.

Figure 6. Downregulation of fluorescence intensity of FKBP12.6/12 in neonatal rat cardiomyocytes was induced by isoproterenol and was blunted by selective ET_A antagonist PD156707, ET_B antagonist IRL1038, dual ET antagonist CPU0213 and argirhein. (A) Control; (B) ISO (1 μ mol/L); (C) ISO+PD156707 (0.01 μ mol/L); (D) ISO+PD156707 (0.1 μ mol/L); (E) ISO+PD156707 (1 μ mol/L); (F) ISO+IRL1038 (0.01 μ mol/L); (G) ISO+IRL1038 (0.1 μ mol/L); (H) ISO+IRL1038 (1 μ mol/L); (I) ISO+AR (0.1 μ mol/L); (J) ISO+AR (1 μ mol/L); (K) ISO+AR (10 μ mol/L); (L) ISO+CPU0213 (0.01 μ mol/L); (M) ISO+CPU0213 (0.1 μ mol/L); (N) ISO+CPU0213 (1 μ mol/L); (O) The gray density of immunocytochemistry of FKBP12.6. $n=6$. Mean $\pm$ SD. $^cP<0.01$ vs control. $^eP<0.05$, $^fP<0.01$ vs ISO. $^gP<0.05$ vs PD156707. $^hP<0.05$, $^iP<0.01$ vs IRL1038.

sity of FKBP12.6/12 in single incubated cardiomyocytes was bright in untreated cells but appeared to be faint after exposure to ISO for 18 h (Figure 6A, 6B, 6O), clearly indicating the downregulation/dissociation of FKBP12.6/12 by ISO in cardiomyocytes. Both ET_A and ET_B antagonists (PD156707 and IRL1038, respectively) enhanced the fluorescence intensity of cardiomyocytes in a dose-dependent manner (Figure 6C–6H, 6O). The effect of IRL1038 was impressive but less potent. Additionally, the novel compound argirhein was shown to increase the fluorescence intensity of FKBP12.6/12 at concentrations of 10^{-6} and 10^{-5} mol/L (Figure 6J, 6K, 6O). The dual endothelin receptor antagonist CPU0213 was potent in escalating the fluorescence in single cardiomyocytes and the mean

gray value was higher than that of either PD156707 or IRL1038 (Figure 6L–6N, 6O). These results indicate that ISO-induced FKBP12.6/12 downregulation is modulated dramatically by either ET_A or ET_B and that argirhein significantly reversed the changes in FKBP12.6/12 expression induced by ISO, but argirhein was less effective than PD156707 and CPU0213.

Discussion

Profound stimulation of β -receptors is commonly found in hearts that manifest worsening of arrhythmogenesis and declines in heart performance^[20, 21]. It is believed that ISO worsens cardiac function and increases the risk of severe ventricular tachyarrhythmias. These activities are thought to

be related to an impairment of the calcium handling protein FKBP12.6/12 at the SR. Calcium leaks during diastole result from the downregulation of FKBP12.6/12, which loses the ability to control the calcium releasing channels (RyR2). This leads to slow down repolarization followed by prolonged APD (action potential duration), and causes EAD (early after depolarization), which predisposes the heart to tachyarrhythmias^[12]. A significant reversal of the deterioration of cardiac function and arrhythmogenic trends of affected hearts can be achieved through rescuing the depressed the FKBP12.6/12 level by blocking the activity of the ET receptors using CPU0213, a dual endothelin receptor antagonist^[12, 19, 22]. In addition to ET antagonists, there are some compounds that specifically normalize abnormal RyR2 (and FKBP12.6/12) resulting from calcium and potassium channel blocking agents such as JTV519^[23] and CPU86017, which was developed at our laboratory^[24, 25].

The downregulation of FKBP12.6/12 induced by ISO is not a single event and is associated with an increase in other inflammatory factors, including ET, ROS, leptin, and an activated NADPH oxidase, in mediating the adverse effects of strong β -adrenoceptor stimulation in the heart^[18, 19, 25]. In this regard, the role of ISO in activating β -receptors to downregulate FKBP12.6 is mediated by ET receptors and related pathways. In the present study we show that a selective antagonist of ET_A and ET_B rescue the downregulation of FKBP12.6/12 individually, in which ET_B exerted an effect comparable to that of ET_A. This phenomenon suggests that ET_B is actively implicated in the hyperadrenergic state and triggers events that worsen cardiac performance and ventricular tachyarrhythmias. In cardiac fibroblasts incubated with ISO, ET_B plays a minor role in the upregulation of Cx43, MMP-2, MMP-9, and NADPH oxidase relative to ET_A^[18]. We found that CPU0213 is more effective than PD156707 and IRL-1038 in modulating FKBP12.6/12, which is consistent with the findings in a previous study^[18]. Our findings are supported by evidence that ET_A and ET_B are located on the sympathetic nerve ending in the myocardium and control release of norepinephrine individually^[26].

Rhein, an active component of *Rheum officinale* Baill, has anti-inflammatory activity and helps to relieve hepatic fibrosis^[16]. With the activity against inflammatory factors in the kidney, rhein has been shown to be effective in treating diabetic nephropathy either as a single therapy^[27, 28] or in combination with benazepril^[29]. The solubility of rhein is low, and chemical modification is encouraged to improve its chemical properties. Di-acetyl-rhein (diacerein, diacerhein) was produced by adding two acetyl groups to the moiety and was launched for treating osteoarthritis in Europe^[30]. Rhein functions as the active metabolite of diacerein in treating osteoarthritis by suppressing osteoarthritic chondrocytes and osteoclastic differentiation/survival^[30]. Diacerein suppresses proinflammatory cytokine expression in nonobese diabetic (NOD) mice^[31]. The extracellular matrix activity is modulated by rhein, mediated by inhibiting the ERK and JNK-AP-1 pathways^[32].

Argirhein is a new compound containing two active mol-

ecules, rhein and *L*-arginine. The compound easily dissociates to form the two compounds in the body. As a consequence, the pharmacological effects of argirhein are relevant to the anti-inflammatory activity of rhein. On the other hand, an improvement in endothelial cells could be achieved by releasing *L*-arginine, which is beneficial to the recovery of dysfunctions of the vascular endothelium. A normal vascular endothelium is essential for cardiac function^[7]. Activated ET_A and ET_B which participate in inflammatory reactions have been shown to be inhibited by argirhein.

An increase in ROS, ET and other cytokines is implicated in the events following the application of ISO^[33], and in patients with ventricular tachyarrhythmias, inflammatory factors are critically involved in the pathogenesis, which is triggered by β -receptor activation, of conditions such as hyperthyroidism in which the incidence of cardiac arrhythmias is common, in association with exaggerated stimulation of β -adrenoceptors^[34, 35]. As compared to those happened in acquired heart diseases genetic mutation cover only a small portion of severe arrhythmias, such as arrhythmogenic cardiomyopathy^[36] and arrhythmias in patients with Brugada syndrome^[37], indicating that inflammatory factors are likely the major causal factors implicated in the affected myocardium responsible for arrhythmogenesis.

In conclusion, the downregulation of FKBP12.6/12, a calcium-modulating protein at the sarcoplasmic reticulum, is a key event involved in the worsening of heart dysfunction and in the arrhythmogenesis caused by stress related to β -receptor stimulation. We demonstrated that both ET_A and ET_B played individual roles in mediating the downregulation of FKBP12.6/12 caused by ISO application. ET_B is definitely an active participant in this regard. We also showed for the first time that argirhein, a new compound containing rhein and *L*-arginine, shared the activity attenuating the ISO-induced downregulation of FKBP12.6/12 with ET blockers. Argirhein is potential drug for use in relieving stress related exacerbation of cardiac failure and arrhythmias by rescuing downregulation of FKBP12.6/12.

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Author contribution

Guo-lin ZHANG conducted the project, processed the data and prepared the manuscript. De-zai DAI and Tao XI designed the project, discussed the mechanisms underlying the results and revised the manuscript. Xiao-dong CONG and Yun ZHANG investigated the new compound argirhein and discussed the data. Yin DAI supervised the experiment and reviewed the collected data.

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