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Reversal of isoproterenol-induced downregulation of phospholamban and FKBP12.6 by CPU0213-mediated antagonism of endothelin receptors[1]

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Key words

heart failure; endothelin receptor antagonists; phospholamban; FKBP12.6; propranolol; CPU0213

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Abstract

Aim: The downregulation of phospholamban (PLB) and FKBP12.6 as a result of β-receptor activation is involved in the pathway(s) of congestive heart failure. We hypothesized that the endothelin (ET)-1 system may link to downregulated PLB and FKBP12.6. **Methods:** Rats were subjected to ischemia/reperfusion (I/R) to cause heart failure (HF). 1 mg/kg isoproterenol (ISO) was injected subcutaneously (sc) for 10 d to worsen HF. 30 mg/kg CPU0213 (sc), a dual ET receptor (ET_AR/ET_BR) antagonist was given from d 6 to d 10. On d 11, cardiac function was assessed together with the determination of mRNA levels of ryanodine receptor 2, calstabin-2 (FKBP12.6), PLB, and sarcoplasmic reticulum Ca^{2+}-ATPase. Isolated adult rat ventricular myocytes were incubated with ISO at 1×10^{-6} mol/L to set up an *in vitro* model of HF. Propranolol (PRO), CPU0213, and darusentan (DAR, an ET_AR antagonist) were incubated with cardiomyocytes at 1×10^{-5} mol/L or 1×10^{-6} mol/L in the presence of ISO (1×10^{-6} mol/L). Immunocytochemistry and Western blotting were applied for measuring the protein levels of PLB and FKBP12.6. **Results:** The worsened hemodynamics produced by I/R were exacerbated by ISO pretreatment. The significant downregulation of the gene expression of PLB and FKBP12.6 and worsened cardiac function by ISO were reversed by CPU0213. *In vitro* ISO 1×10^{-6} mol/L produced a sharp decline of PLB and FKBP12.6 proteins relative to the control. The downregulation of the protein expression was significantly reversed by the ET receptor antagonist CPU0213 or DAR, comparable to that achieved by PRO. **Conclusion:** This study demonstrates a role of ET in mediating the downregulation of the cardiac Ca^{2+}-handling protein by ISO.

Introduction

Heart failure (HF) is one of the leading causes of morbidity and mortality in our society. The therapeutic prospects remain poor and this may be attributed to an inadequate understanding of the multiple and complex mechanisms underlying HF. In failing hearts, some molecular events which worsen cardiac performance and are predisposed to developing life threatening-arrhythmias are likely to be related to the overactivation of β-adrenergic receptors (β-AR)[1,2]. However, the mechanisms underlying the consequences of β-receptor activation in HF remain to be clarified. Protein kinase A (PKA) phosphorylation as the consequence of β-AR activation significantly affects proteins of the intracellular calcium handling system[3], including ryanodine receptor type 2 (RyR2), FKBP12.6 (calstabin2), phospholamban (PLB), and sarcoplasmic reticular Ca^{2+} ATPase 2a (SERCA2a) in the sarcoplasmic reticulum (SR). The application of isoproterenol (ISO) which significantly worsens cardiac performance is considered to produce PKA hyperphosphorylation[4]. PKA hyperphosphorylation increases diastolic $[Ca^{2+}]_i$ which seriously disturbs cardiac function. A calcium leak from the Ca^{2+} release channel RyR2 where FKBP12.6 is dissociated by downregulation and a reduction in the uptake activity of SERCA2a contribute to elevated diastolic calcium levels. The normal function of SERCA2a is dependent on its modulating

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protein PLB which is markedly affected by PKA phosphorylation. Thus, the normal expression of FKBP12.6 and PLB is crucial to maintaining cardiac performance by keeping low Ca^{2+} levels at diastole and sufficient calcium stores in the SR for systolic release under physiological conditions.

In general, depressed SERCA2a and RyR2 and their modulating proteins PLB and FKBP12.6 are observed in chronic HF (CHF)[5,6], resulting in an increase in diastolic $[Ca^{2+}]_i$. The modulating proteins FKBP12.6 and PLB are abnormal in compromised hearts. Cardiac insufficiency can be induced by a mutated PLB[7]. Acute cardiac failure caused by ischemia/reperfusion procedure (I/R) is associated with depressed Ca^{2+} uptake and Ca^{2+} release in association with the down-regulation of SERCA2a and PLB and the upregulation of PKA-mediated phosphorylation which are reversed by β-blockers[8]. The reversal of the abnormal expression of FKBP12.6 has also been targeted to relieve the molecular disturbance in HF[9]. Thus, it was of interest to investigate a reversal of the abnormal expression of FKBP12.6 and PLB other than by β-blockade to increase our understanding of the molecular mechanisms underlying HF.

A role for endothelin (ET)-1 has been implicated. HF and ET blocking agents are considered candidates for therapeutic uses in the treatment of HF[10]. The upregulation of the ET pathway is involved in the pathological process of HF, and ET receptor antagonism has been successful in treating acute HF[10,11], but remains controversial in CHF[12,13]. The application of the dual ET receptor antagonist CPU0213 relieves cardiac dysfunction in diabetic cardiomyopathy[14] and HF produced by coronary I/R[15], together with an improvement in the calcium handling system in the myocardium. Excessive ET may stimulate the myocardium to produce more reactive oxygen species (ROS); conversely, an increase in ROS facilitates ET pathway activation[16,17] to further adversely impact the cardiovascular system.

The downregulation of FKBP12.6 and PLB in association with compromised cardiac performance in diabetic hearts, which could be related to the stimulation of β-receptors, is significantly attenuated by the ET receptor antagonist CPU0213. Thus, we hypothesized that ISO, which dramatically downregulates the expression of FKBP12.6 and PLB, worsens cardiac insufficiency, and may cause activation of the ET pathway, an intermediate event in the downstream events following vigorous stimulation of β-AR. It was of interest to further study the molecular changes relating to HF by examining whether the downregulation of the expression of FKBP12.6 and PLB and worsened cardiac dysfunction by ISO could be reversed by the application of a dual ET_AR/ET_BR antagonist CPU0213.

Materials and methods

Animals Male, 12-week old Sprague-Dawley rats, weighing 220–250 g were purchased from the Animal Center of Nanjing Medical University (Nanjing, China). The animal handling was in accordance with the Provincial Regulations of Animal Care and Use in Jiangsu Province (Nanjing, China).

Chemicals ISO was purchased from Shanghai Hefeng Medicine (Shanghai, China); CPU0213 (0213, dajisentan) and darusentan (DAR) were from the Department of Medicinal Chemistry of the China Pharmaceutical University (Nanjing, China); and Propranolol (PRO) was from Sigma (St Louis, MO, USA).

Acute HF and ISO The rats were divided into 3 groups with 10 rats in each: (i) normal; (ii) ISO: administration with 1 mg/kg ISO subcutaneously (sc) on d 1–10; and (iii) CPU0213: 1 mg/kg ISO on d 1–10 and 30 mg/kg CPU0213 (sc) on d 6–10. On d 11, the rats were placed under urethane anesthesia and the main left coronary artery was occluded after chest opening. Acute cardiac failure was produced by 10 min occlusion of the coronary artery (ischemia) followed by reperfusion for 10 min (I/R). Cardiac function was assessed by inserting a catheter into the left ventricular chamber to measure the systolic (LVSP) and diastolic function (LVEDP). Data were collected for 10 rats in each group.

Semiquantitative determination of FKBP12.6 and PLB mRNA by RT-PCR The total mRNA was extracted from the homogenate of the frozen left ventricle using Trizol reagent and reversely transcribed to cDNA by using AMV reverse transcriptase (Promega, USA) according to previous publications and the manufacturer's instructions[14,18]. The total volume of the PCR reaction was 25 μL: 1 μL cDNA, 2 mmol/L $MgCl_2$, 20 mmol/L each dNTP, 0.2 nmol/L each primer, 2 U DNA *Taq* polymerase, and the accompanied buffer. The cDNA was amplified under the following conditions: initial denaturation at 94 °C for 5 min, then cycling and denaturation at 94 °C for 40 s, annealing for 40 s extending at 72 °C for 1 min. The annealing temperature and cycle number of RyR2, FKBP12.6, PLB, SERCA2a, and GAPDH were 58 °C, 36 cycles; 61 °C, 32 cycles; 63 °C, 30 cycles; 54 °C, 36 cycles; and 65 °C, 30 cycles, respectively. It was followed by a final extension at 72 °C for 10 min. The specific primers for RyR2 (AF130880) were: sense, 5'-GAATCAGTGAGTTACTGGGCATGG-3' and antisense, 5'-CTGGTCTCTGAGTTCTCCAAAAGC-3'; for FKBP12.6 (D86642): sense, 5'-GTGAAGGCAGGAAGGAA-3' and antisense, 5'-GCAGCCAACAGAAGATAAG-3'; for PLB (NM_022707): sense, 5'-TACCTTACTCGCTCGGC-TATC-3' and antisense, 5'-CAGAAGCATCACAATGATG-

CAG-3'; for SERCA2a (NM_017290): sense, 5'-CCGTATCCGATGACAATG-3' and antisense, 5'-CCAGGCTCCAGGTAGTTT-3'; and for GAPDH: sense, 5'-GCTGGGGCTCACCTGAAGG-3' and antisense, 5'-GGATGACCTTGCC CACAGCC-3', respectively.

The amplification products were separated by agarose gel electrophoresis (2%), stained with ethidium bromide, visualized under UV light, and digitally scanned (Syngene, England). Band density was determined by a gel imaging analysis system (Genegenus, Syngene, England), and the relative density of each DNA band was obtained by dividing that of the GAPDH bands.

Isolation of rat ventricular myocytes The rat ventricular myocytes were isolated from adult, male Sprague-Dawley rats as described previously[19]. Briefly, the rats were killed by an intraperitoneal (ip) injection of a lethal dose of pentobarbital (100 mg/kg), then the chest cavity was opened and the hearts were excised. The excised hearts were retrogradely perfused at 7 mL/min through the aorta, first for 5 min with Ca^{2+}-free Tyrode's solution composed of (in mmol/L): 135.0 NaCl, 5.4 KCl, 2.0 $MgSO_4$, 0.33 NaH_2PO_4, 10.0 glucose, and 10.0 HEPES (pH 7.4) at 37 °C, then with Ca^{2+}-free Tyrode's solution containing collagenase type II (0.33 mg/mL) and protease (0.16 mg/mL) for 12 min, and finally with Tyrode's solution containing 0.2 mmol/L $CaCl_2$ for 6 min. The ventricles of the digested heart were then cut into small cubes that were subjected to gentle agitation to dissociate the cells. The freshly dissociated cells were stored at room temperature in Tyrode's solution containing 0.2 mmol/L $CaCl_2$.

ISO stimulates cardiomyocytes The ventricular myocytes were isolated as described earlier, plated onto laminin-coated coverslip chambers, and cultured at 37 °C in MEM/EBSS serum-free medium. Four hours later, drugs were added into chambers to assess the change of PLB expression as follows: (i) control (0.2 μL dissolvent); (ii) ISO (1×10^{-6} mol/L ISO+0.2 μL dissolvent); (iii) PRO-5 and PRO-6 (1×10^{-6} mol/L ISO+1×10^{-5} mol/L or 1×10^{-6} mol/L PRO); (iv) DAR-5 and DAR-6 (1×10^{-6} mol/L ISO+1×10^{-5} mol/L or 1×10^{-6} mol/L DAR); and (v) 0213-5 and 0213-6 (1×10^{-6} mol/L ISO+1×10^{-5} mol/L or 1×10^{-6} mol/L CPU0213). In a separate experiment, changes in the FKBP12.6 expression in cardiomyocytes were investigated with PRO and dajisentan (CPU0213) at 1×10^{-5} mol/L and 1×10^{-6} mol/L added to the medium and ISO at 1×10^{-6} mol/L.

Immunocytochemistry assay of PLB Twenty-four hours after drug incubation, the ventricular myocytes were fixed using 4% paraformaldehyde for 15 min at 37 °C. The fixed cells were rinsed 3 times in phosphate-buffered saline (PBS) and then incubated for 30 min with 2% bovine serum albumin in PBS to reduce non-specific binding. After overnight incubation (at 4 °C) with the primary antibody against PLB (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the cells were rinsed 4 times with PBS, thereafter, the secondary antibody conjugated with fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, USA) was added for 2 h at room temperature. Following a further 4 washes, the stained cells were imaged under fluorescence microscopy. The negative control, which received identical treatment with the exception of the primary antibody treatment, was conducted to exclude non-specific staining.

For the convenience of data analyses, the color images were converted to red- and blue-filtered, gray-scale images by ImagePro 5.0 software. In the gray-scale images the "green" staining of PLB was highlighted above a uniform background. This step resulted in the narrow and peaked gray density distribution of pixels in the cardiomyo-cytes. The gray density represented positive-stained pixels. The darkest pixel (black) was set to gray value 0 and the brightest pixel (white) was set to gray value 255, thus, the pixels were set to gray images between 20–120.

Western blot analysis of PLB and FKBP12.6 After 24 h incubation, the ventricular myocytes were then washed twice with cold PBS (137 mmol/L NaCl, 1.47 mmol/L KH_2PO_4, and 8.9 mmol/L Na_2HPO_4, pH 7.4) and put into 500 μL lysing buffer containing 50 mmol/L Tris-HCl, 1% Triton X-100, 150 mm NaCl, 1 mmol/L EDTA, 0.5% SDS, and 1 mmol/L phenylmethylsulfonyl fluoride. The homogenates were centrifuged for 10 min at 10 000×*g* at 4 ºC and the supernatants were collected. The cell lysates (40 mg) were analyzed by 10% SDS-PAGE at 100 V for 2 h. After transfer, the nitrocellulose membranes were incubated in a blocking buffer (50 mmol/L Tris-HCl, 200 mmol/L NaCl, 0.2% Tween 20, and 5% non-fat dried milk) for 1 h at room temperature, followed by 2 h in the same buffer containing 1% non-fat dried milk with a polyclonal antibody raised against PLB and FKBP12.6 (Santa Cruz Biotechnology, USA). The membranes were washed with the same buffer without milk and then incubated for 1 h with the rabbit antigoat antibody (Wuhan Boster Biological Technology, Wuhan, China). After washing 3 times for 15 min each time, the immunoreactive bands were visualized by enhanced chemiluminescence detection reagent (Wuhan Boster Biological Technology, China) and quantified by densitometry as described earlier.

Statistical analysis The fluorescence images were analyzed by ImagePro 5.0 software. Data are presented as mean±SD or mean±SEM. The homogeneity of the data was tested by one-way ANOVA and the differences between specific means were tested for significance by Bonferroni's multiple comparison tests. A difference between 2 means

was considered statistically significant when $P<0.05$.

Results

Worsening cardiac dysfunction and ventricular fibrillation (VF) rate by ISO Cardiac dysfunction was evaluated by measuring intracardiac pressure following I/) episodes in normal and ISO-treated rat hearts. A decrease in systolic (LVSP, Figure 1A) and diastolic (LVEDP, Figure 1B) performance was found by I/R procedure relative to the control. Acute HF during I/R was worsened in the ISO-treated rats with sharply depressed systolic and diastolic function relative to I/R in the normal rats ($P<0.05$). Thus, a worsened HF model was established attributed to ISO medication which strongly stimulates b-receptors. In the CPU0213 group the impaired cardiac performance was attenuated relative to the

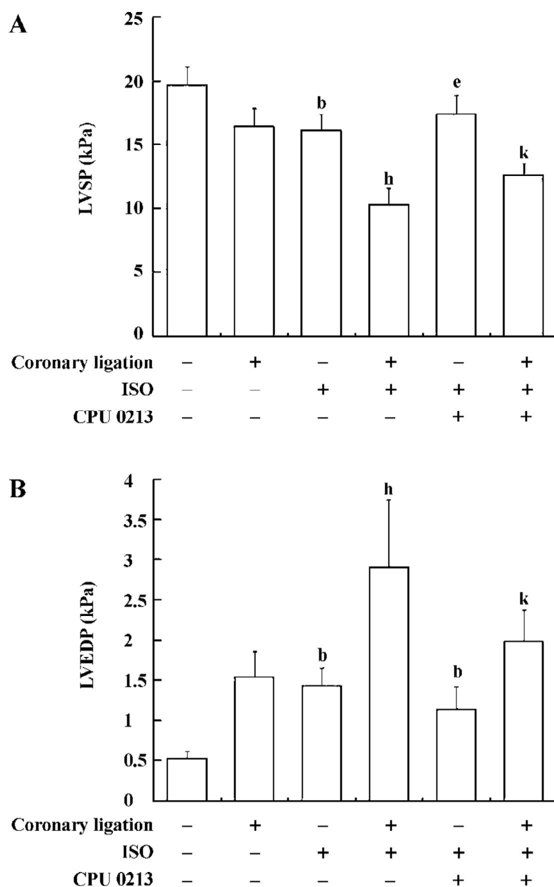

Figure 1. Changes in hemodynamics before and after coronary I/R in the normal and ISO-treated rat hearts. ISO (1 mg/kg) was injected sc on d 1–10 and 30 mg/kg CPU0213 (sc) commenced on d 6–10. n=10. Mean±SD. $^{b}P<0.05$ *vs* the normal rat hearts before ligation; $^{e}P<0.05$ *vs* the ISO-treated rat hearts before ligation; $^{h}P<0.05$ *vs* the normal rat hearts after ligation; $^{k}P<0.05$ *vs* the ISO-treated rat hearts after I/R. (A) LVSP; (B) LVEDP.

ISO group, indicating that the adverse impact by ISO on the cardiac function was significantly reversed ($P<0.05$) by antagonism of ET receptors by CPU0213.

Downregulation of mRNA of the calcium handling system by ISO The exacerbation of cardiac dysfunction was produced by ISO subsequent to I/R. The downregulation of RyR2, FKBP12.6, PLB, and SERCA2a (Figure 2A–2D) was established by RT-PCR to be significant ($P<0.01$) relative to the control, respectively. CPU0213 intervention significantly reversed the downregulation of the expression of the calcium handling system compared to the ISO group ($P<0.01$).

Downregulation of PLB by ISO *in vitro* The incubation of the adult rat ventricular myocytes with ISO at 1×10^{-6} mol/L was conducted to establish an in vitro of downregulation of PLB. After incubation of the second antibody conjugated with FITC, the PLB proteins in the cardiac myocytes were flamed with green fluorescence (Figure 3). Following 24 h incubation with ISO, the green fluorescence was reduced significantly and the mean gray value of the cardiomyocytes was reduced to 31.7±7.1, from 57.6±6.0 for the control group ($P<0.01$; Figure 4). ISO suppressed the peak of gray density distribution in the cardiomyocytes relative to the control (Figure 4A, 4B).

Reversal of PLB downregulation by PRO *in vitro* It was of interest to identify whether the downregulation of PLB by ISO was due to the activation of β-AR. The altered gray images of PLB in isolated cardiomyocytes were completely reversed by PRO at either 1×10^{-5} mol/L (51.45±1.8, $P<0.01$) or 1×10^{-6} mol/L (47.98±7.5, $P<0.05$), respectively. In this cellular model, the downregulation of PLB by ISO was subsequent to an overactivation of the β-AR *in vitro* (Figure 4C, 4D).

Reversal of PLB downregulation by ET receptor antagonism *in vitro* To further investigate whether ET receptor antagonism could reverse the downregulation of PLB by ISO, the effects were compared with PRO *in vitro*. A selective ET_A blocker DAR at 1×10^{-5} mol/L and 1×10^{-6} mol/L significantly elevated the mean gray values in ventricular myocytes to 59.49×1.0 ($P<0.01$) and 47.38×7.5 ($P<0.05$), respectively (Figure 5A, 5B), relative to the ISO group, and the pattern of the images was restored towards the control levels.

It was also interesting to determine whether the reversal of the downregulation of PLB could also be achieved by a dual ET_A/ET_B blocker CPU0213 *in vitro*. Incubation with CPU0213 at 1×10^{-5} mol/L and 1×10^{-6} mol/L dramatically upregulated the PLB protein levels, as an increase in immunostaining levels (54.61±3.2, $P<0.01$ and 47.10±3.5, $P<0.05$) relative to the ISO group (Figure 5C, 5D). The protection of cardiomyocytes from the downregulation of PLB in the presence of ISO was significant by either DAR or CPU0213 *in*

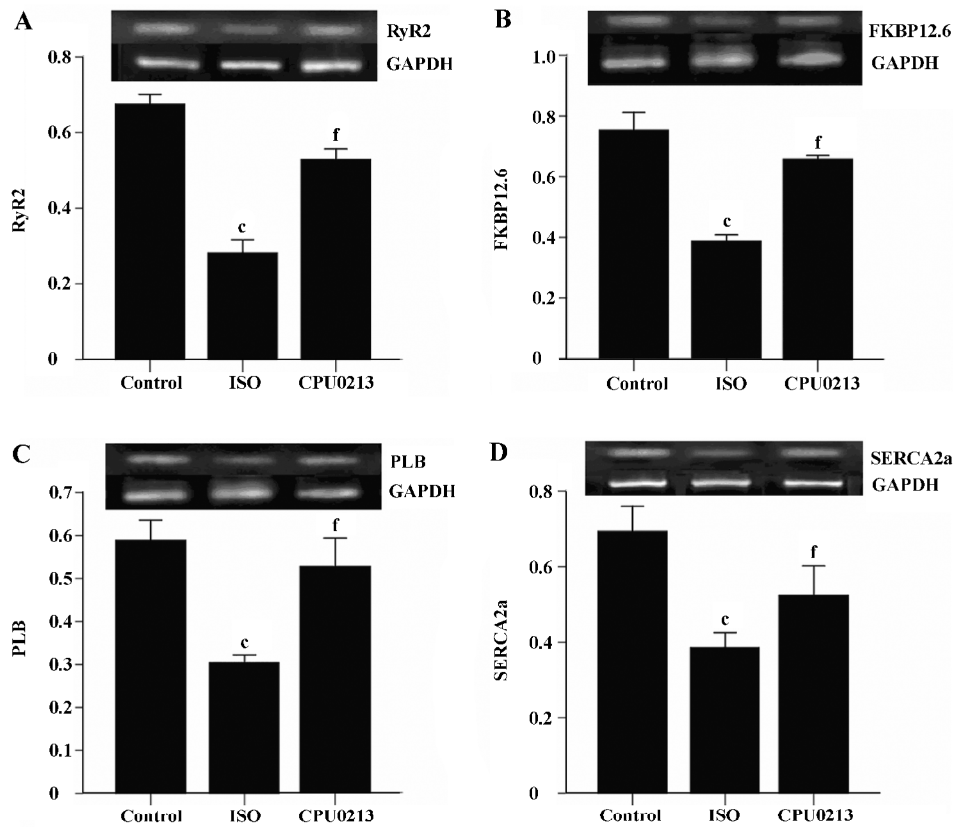

Figure 2. mRNA expression of the calcium handling system in the SR of the myocardium subjected to I/R episode and under stimulation of ISO. n=4. Mean± SEM. [b]P<0.05, [c]P<0.01 *vs* the normal rat hearts. [e]P<0.05, [f]P<0.01 *vs* the ISO-treated rat hearts. (A) RyR2; (B) FKBP12.6; (C) PLB; (D) SERCA2a.

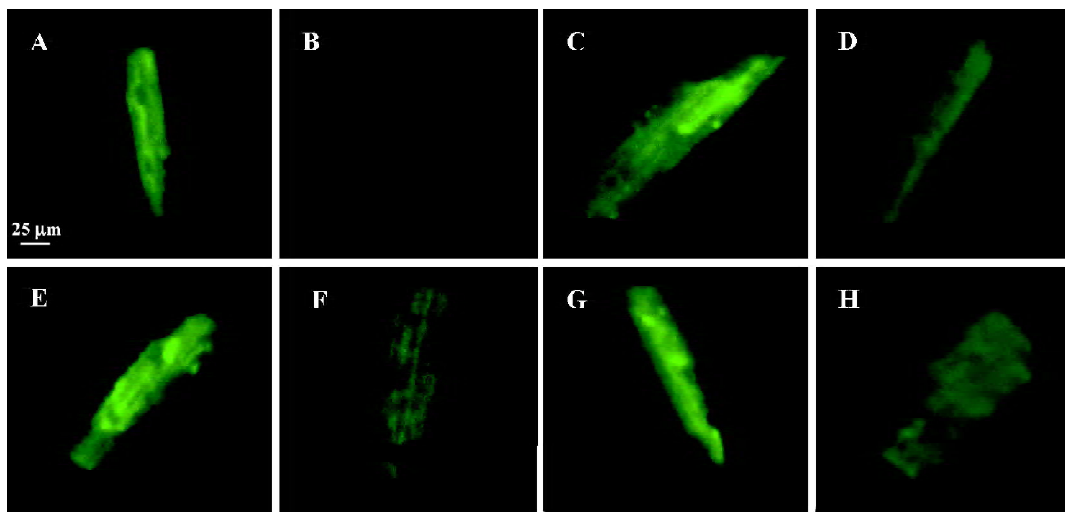

Figure 3. Immunostaining of PLB affected by ISO in the adult rat cardiomyocytes. A cellular model of downregulation of PLB by ISO is established with fluorescent images observed under a fluorescence microscope (scale bar=25 μm). ISO-induced phosphorylation of PLB was intervened by 2 concentrations of drugs. Cells with representative images are listed. (A) control; (B) ISO (1×10^{-6} mol/L); (C) ISO+PRO (1×10^{-5} mol/L); (D) ISO+PRO (1×10^{-6} mol/L); (E) ISO+DAR (1×10^{-5} mol/L); (F) ISO+DAR (1×10^{-6} mol/L); (G) ISO+CPU0213 (1×10^{-5} mol/L); (H) ISO+CPU0213 (1×10^{-6} mol/L).

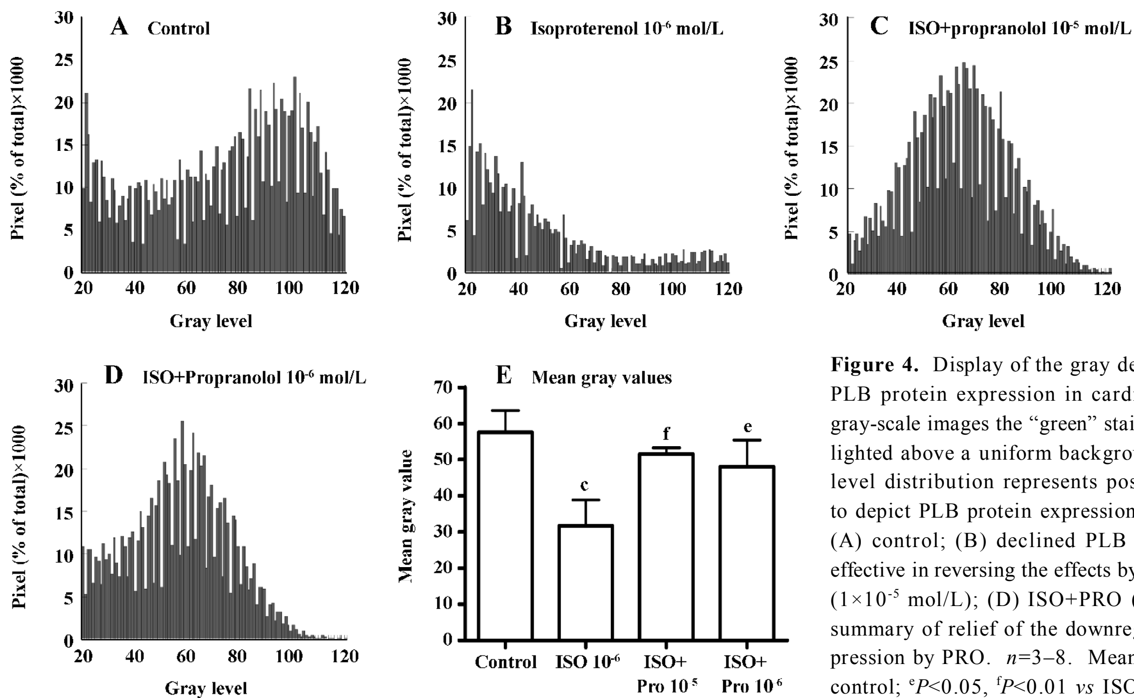

Figure 4. Display of the gray density distribution of PLB protein expression in cardiomyocytes. In the gray-scale images the "green" staining of PLB is highlighted above a uniform background. A peaked gray level distribution represents positive-stained pixels to depict PLB protein expression in cardiomyocytes. (A) control; (B) declined PLB by ISO. PRO was effective in reversing the effects by ISO; (C) ISO+PRO (1×10^{-5} mol/L); (D) ISO+PRO (1×10^{-6} mol/L); (E) summary of relief of the downregulation of PLB expression by PRO. n=3–8. Mean±SEM. $^{c}P<0.01$ *vs* control; $^{e}P<0.05$, $^{f}P<0.01$ *vs* ISO.

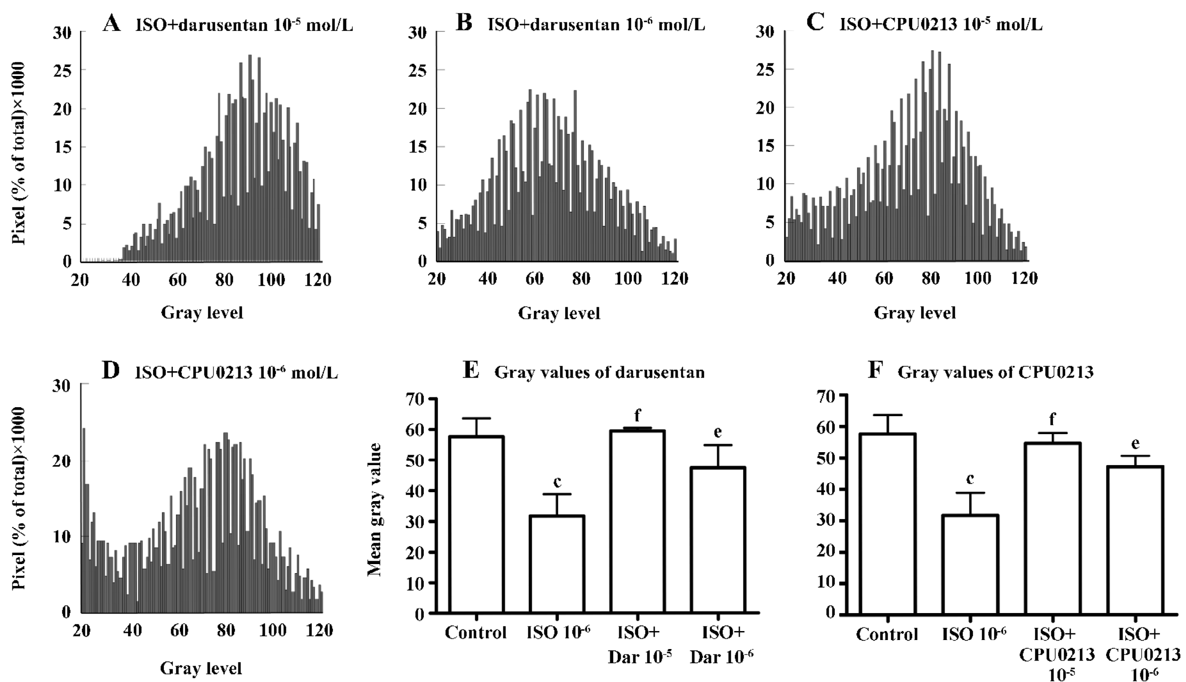

Figure 5. PLB protein downregulation by immunostaining in cardiomyocytes by ISO was reversed by DAR and CPU0213(dajisentan). (A) ISO+DAR (1×10^{-5} mol/L); (B) ISO+DAR (1×10^{-6} mol/L); (C) ISO+CPU0213 (10^{-5} mol/L); (D) ISO+CPU0213 (1×10^{-6} mol/L); (E) summary of relief in the downregulation of the PLB protein by DAR; (F) summary of relief of the downregulation of the PLB protein by CPU0213. n=3–8. Mean±SEM. $^{c}P<0.01$ *vs* control. $^{e}P<0.05$, $^{f}P<0.01$ *vs* ISO.

vitro (Figure 5E, 5F).

Reversal of PLB and FKBP12.6 protein by Western blotting Data from Western blots present further proof of the downregulation of PLB protein levels by ISO *in vitro*. After incubation of 1×10^{-6} mol/L ISO for 24 h, there was a 39% depression of the PLB protein (P<0.01) relative to the control, and the downregulation of PLB was effectively reversed by either PRO or CPU0213 at 1×10^{-5} mol/L (P<0.01) and 1×10^{-6} mol/L (P<0.05), respectively (Figure 6A).

A dramatic downregulation of FKBP12.6 was also found in the cardiac myocytes stimulated by ISO *in vitro*. The protein level of FKBP12.6 decreased by 48% (P<0.01) compared to the control, and the downregulation of the FKBP12.6 protein was restored by PRO at 1×10^{-5} mol/L (P<0.01) and 1×10^{-6} mol/L (P<0.05). The ET receptor antagonism of CPU0213 significantly elevated the FKBP12.6 protein at both 1×10^{-5} mol/L (P<0.01) and 1×10^{-6} mol/L (P<0.05), respectively, relative to the ISO group. Both the β-blocker and ET receptor antagonist can reverse the downregulation of FKBP12.6 by ISO (Figure 6B).

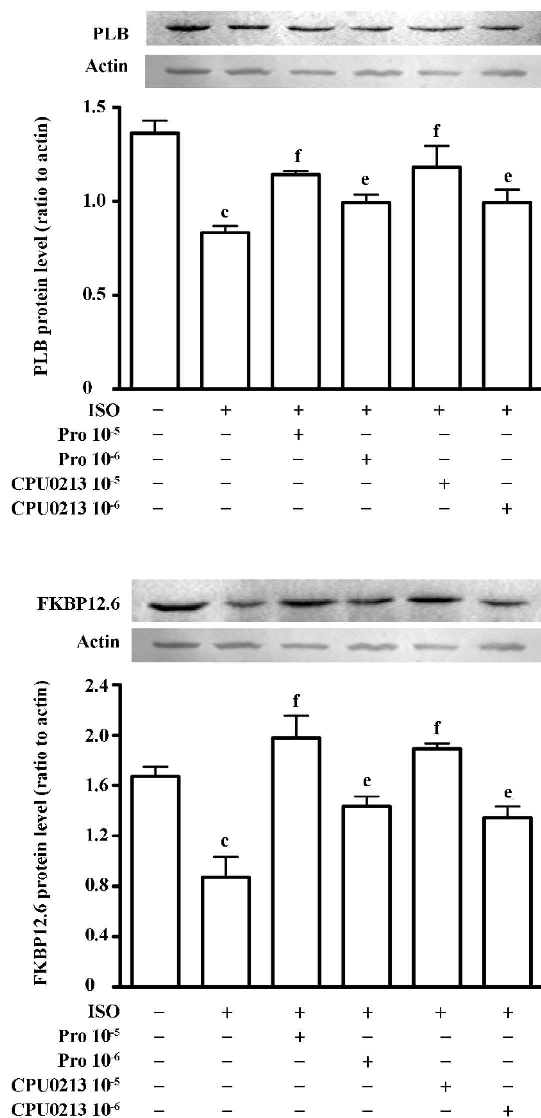

Figure 6. Western blot analysis of PLB and FKBP12.6 proteins was downregulated by ISO and reversed by PRO and CPU0213 in isolated adult rat cardiomyocytes. PLB (A) and FKBP12.6 (B). n=3–8. Mean±SEM. [c]P<0.01 *vs* control. [e]P<0.05, [f]P<0.01 *vs* ISO.

Discussion

Despite extensive investigations, the molecular events and the mechanisms underlying the progression of HF remain unclear. In failing hearts, the cardiac adrenergic receptor signaling pathway is activated, and the most striking target for the treatment of HF is directed to the downstream events of β-AR activation[20]. Molecular aspects of HF have been focused at the depressed expression of FKBP12.6[9] and SERCA2a[6,21] which are potential therapeutic targets. Changes in the downstream signaling pathways (RyR2, FKBP12.6, SERCA2a, PLB, *etc*) in response to β-adrenergic stimulation are also considered to be important targets for future treatments of HF[22]. In the present study, cardiac failure produced by I/R procedure is exacerbated by ISO treatment which also produces significant downregulation in the mRNA expression of RyR2, FKBP12.6, PLB, and SERCA2a in the myocardium *in vivo*. These changes are attenuated by CPU0213 which is a dual ET receptor antagonist[15].

The overactivation of the sympathetic nervous system and the β-AR are found in congestive HF where the expression of PLB and SERCA2a is downregulated[23]. The worsened cardiac dysfunction following ISO treatment correlates well with a marked reduction in the expression of PLB and SERCA2a, possibly resulting from hyperphosphorylation by PKA in response to the overstimulation of β-AR. PKA phosphorylation of PLB under normal conditions releases its sustained suppression of SERCA2a, resulting in elevated activity of SERCA2a in response to an accelerated heart rate in a shortened cardiac cycle. In the presence of excess of ISO, the marked downregulation of both SERCA2a and PLB is produced in the myocardium, which is in agreement with previous studies[24].

The RyR2/calcium release channel in the SR comprises a macromolecular complex in association with FKBP12.6 (calstabin2). FKBP12.6, an 11.8 kDa cis-trans peptidyl-prolyl isomerase (apparent molecular mass 12.6 kDa), stabilizes the

closed state of the RyR2 channel at diastole, and FKBP12.6 can be targeted for improving cardiac performance[25]. However, the underlying mechanism of this regulation has not been fully clarified. It remains to be established whether the expression of RyR2, FKBP12.6, SERCA2a, and PLB is mediated by the activation of ET receptors in the process following the stimulation of β-receptors.

Cardiac dysfunction exaggerated by ISO in association with the downregulation of the calcium handling system is alleviated by ET receptor blockade *in vivo*. The beneficial effects may be explained by an indirect compensatory response via a reflex or some humoral factors rather than a direct involvement in the downstream events of the stimulated β-receptors. An *in vitro* assessment of this possibility has been carried out. ISO incubated with cardiomyocytes *in vitro* produces overactivity of β-receptors and the downregulation of PLB and FKBP12.6 proteins. The present study of *in vitro* mechanisms provided the support that the ET pathway is directly linked to the downregulation of PLB by the activation of β-AR. Interestingly, when ET receptors are blocked by either the ET_AR blocker DAR or ET_AR/ET_BR blocker CPU0213, a reversal of the downregulated protein expression of PLB and FKBP12.6 is equally significant. This suggests that the ET_AR is involved mainly in the response to β-receptor stimulation. Additionally, the protein expression of PLB and FKBP12.6 is downregulated by ISO in cultured myocytes *in vitro* and the downregulation of PLB and FKBP12.6 in the presence of ISO is blocked by CPU0213, the effects of which are comparable to PRO. The present study provides evidence to support the thesis that the downregulation of the calcium handling system in the presence of β-receptor stimulation is at least partly mediated by the activation of the ET pathway. Thus, ET receptor antagonism is effective in treating HF[16,27] and is of potential therapeutic significance in preventing patients from sudden cardiac death[26,28]. The present study may offer more information in the molecular events relating to the downregulation of RyR2 and FKBP12.6 in HF[26].

The activation of the ET pathway may initiate oxidative stress, which in turn releases more ET-1 to affect the cardiovascular system; a positive therapeutic outcome of an antagonism of the ET receptor also results from antioxidative activities[14,16]. Additionally, a relief of cardiac failure by ET receptor antagonists also corresponds to an improvement of mitochondrial respiratory complex activities in the myocardium[27].

The findings of this study both *in vivo* and *vitro* is that β-AR activation, which induces the downregulation of the calcium modulating system, is at least partly relayed by an activated ET pathway. The blockade of ET receptors reverses the abnormal expression of the calcium handling system in the SR and the worsened cardiac function produced by ISO. This study offers direct evidence that ET-1 is actively involved in the downregulation of PLB and FKBP12.6 through β-AR overactivation and that this mode of action is consistent with an attenuation of heart failure by ET antagonism.

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