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Down-regulation of FKBP12.6 and SERCA2a contributes to acute heart failure in septic shock and is related to an up-regulated endothelin signalling pathway

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Abstract

Acute heart failure (AHF) critically affects morbidity and mortality in patients suffering from septic shock. It is hypothesized that AHF is linked to down-regulation of FKBP12.6 (calstabin 2) and SERCA2a (sarco/endoplasmic reticulum Ca^{2+} ATPase 2a), which may be mediated by an activated endothelin (ET) system in the myocardium. The aim of the study was to test whether an attenuation of septic AHF can be achieved by a novel dual endothelin receptor antagonist, CPU0213, in association with up-regulation of FKBP12.6 and SERCA2a in rats. AHF in septic shock was produced by faeces leak from a surgically punctured caecum for 72 h in rats. CPU0213 (30 mg kg^{-1}, s.c., every 12 h, for 3 days) was administered to rats 8 h after the operation. In the untreated model group, survival rate markedly decreased ($P < 0.01$), and the cardiac performance was seriously compromised ($P < 0.01$) relative to control. The AHF was characteristically associated with down-regulated mRNA and protein expressions of FKBP12.6, SERCA2a and PLB (phospholamban). Elevated ET-1 and mRNA abundances of the preproET-1, ECE (endothelin converting enzyme) and ET_A and ET_B receptors in the left ventricular tissue ($P < 0.01$) were found. All abnormalities were reversed significantly following CPU0213 administration. In conclusion, septic AHF is attributed to down-regulation of FKBP12.6 and SERCA2a, which is related to an activated ET system. An endothelin receptor antagonism of CPU0213 significantly improves the cardiac performance by blocking both ET_A and ET_B receptors.

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Introduction

The clinical syndrome of septic shock is complicated by acute heart failure (AHF), which contributes to morbidity and mortality of patients suffering from severe Gram-negative bacillus infection. Molecular mechanisms underlying cardiac dysfunction in AHF have not been evaluated clearly. However, great progress has been achieved in discovering dysfunction of the calcium handling system in sarcoplasmic reticulum (SR) in congestive heart failure (Marks 2003; Olson 2004). Down-regulation of calstabin 2 (FKBP12.6) is one of the key molecular steps implicated in congestive heart failure where an increased diastolic calcium is attributed to phosphorylation of the ryanodine receptor type 2 (RyR2) by PKA (protein kinase A), which eventually produces a down-regulation of FKBP12.6. Thus, an abnormal function of RyR2 results. On the other hand, the activity of SERCA2a (sarco/endoplasmic reticulum calcium ATPase 2a) is responsible for the calcium uptake process into the SR and is under the control of phospholamban (PLB) by PKA phosphorylation. A down-regulation of SERCA2a, which may reflect an increased Ca^{2+} at the diastole, can be found in congestive heart failure. It is not clear whether both down-regulated FKBP12.6 and SERCA2a are simultaneously involved in the AHF of septic shock. However, improvement of cardiac performance has been achieved by modulating expression of FKBP12.6 and SERCA2a in failing hearts (Bers et al 2003; Prestle et al 2003; Reiken et al 2003).

Endothelin-1 (ET-1, ET) is a potent vascular smooth-muscle-constricting and proliferating factor and is also a cytokine contributing to progression of septic shock, together with iNOS (inducible nitric oxide synthase) and the reactive oxygen species (ROS) (Okamoto et al 2000; Muta et al 2003). An increase in ET-1 levels is found in the myocardium with ischaemic lesion and cardiac failure (Iskit & Guc 2003; Wang et al 2004), pulmonary hypertension (Zhang et al 2005a, b), acute renal failure (He et al 2006), cardiac arrhythmias (Xia et al 2006), etc. Clinical

findings show that an elevated plasma level of ET-1 correlates well with severity of cardiac dysfunction in septic shock (Wanecek et al 2000). An activated ET-1 signalling pathway is revealed in septic shock, of which abnormalities in the cardiovascular system are reversed by bosentan (Ishimaru et al 2001). However, it is unknown whether down-regulation of FKBP12.6 is critically involved in AHF of septic shock and is linked to an activated ET system.

A novel endothelin receptor antagonist, CPU0213, blocks ET_A and ET_B receptors similarly to bosentan and the selectivity is slightly improved (Dai et al 2004). It is effective in relieving pulmonary hypertension, ventricular hypertrophy and the extracellular matrix (Liu & Dai 2004; Liu et al 2004).

It was hypothesized that an activated ET signalling pathway may correlate to down-regulation of FKBP12.6, SERCA2a and PLB to promote the progression of AHF in septic shock. It is of interest to explore the effectiveness of reversing molecular events in AHF of septic shock by application of the novel dual endothelin receptor antagonist CPU0213. We targeted down-regulation of FKBP12.6, SERCA2a and PLB in AHF of septic shock to determine whether antagonism of endothelin receptor by CPU0213 could significantly regress down-expression of FKBP12.6 and SERCA2a, and contribute to relieving AHF in septic shock.

Materials and Methods

Animals and procedures

All experiments were carried out by personnel permitted to handle laboratory animals, according to the Guidelines for the Care and Use of Laboratory Animals in Jiangsu Province, People's Republic of China.

Male Wistar rats, 250–280 g, were supplied by the Animal Center of Nanjing Medical University. The rats were randomly assigned into three groups: sham operation, untreated model and CPU0213 groups. Sepsis was induced in rats by caecal ligation and puncture (CLP) procedures according to the literature (Krivoruchko et al 2004). Eight hours after surgery, operated rats were divided randomly into 2 groups: untreated and treated with CPU0213 (30 mg kg^{-1}, s.c., every 12 h, for 3 days; Figure 1). The same volume of normal saline was subcutaneously injected in the sham operation group and untreated model group. Rats were allowed free access to the regular diet and water. The experiment was carried out on the 4^{th} day following operation.

Survival of rats and haemodynamics

Survival rate of rats was monitored within 72 h following surgical operation. At 72 h after operation, the rats were anaesthetized with urethane (1.5 g·kg^{-1}, i.p.) and a polyvinyl chloride catheter was inserted into the left ventricle via the left common carotid, connected to a pressure transducer of a computerized system (MPA-V; Second Military Medical University, China). Blood pressure, left ventricular systolic pressure (LVSP), the maximal rate of left ventricle systolic pressure (LV+dp/dtmax), the minimum rate of left ventricle systolic pressure (LV−dp/dtmin) and left ventricular end diastolic pressure (LVEDP) were continuously monitored and recorded. The heart rate (HR) was monitored by a lead II ECG.

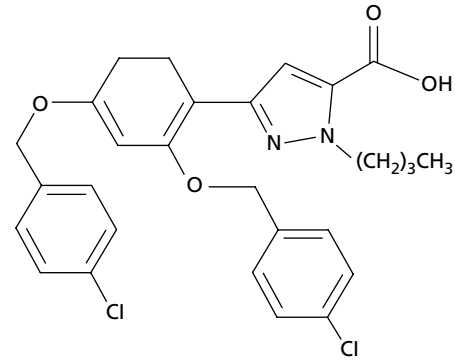

Figure 1 Chemical structure of the novel dual endothelin receptor antagonist CPU0213.

Biochemical assays

Rats were exsanguinated at the end of experiment and the hearts were dissected and weighed. The left ventricles were rapidly trimmed, weighed, snap-frozen in liquid nitrogen and stored at −70°C until further processing. The ratio of whole heart weight to body weight ratio (HW/BW) and left ventricle weight (including the septum) to body weight (LVW/BW) in milligrams per gram were evaluated as an index of cardiac hypertrophy. The activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and levels of malondialdehyde (MDA), nitric oxide (NO) and iNOS in the left ventricle were measured according to the directions of the reagent kits (Jiancheng Bio-engineering Company, Nanjing, China). ET-1 levels in the LV tissue were measured by radioimmunoassay as described previously (Voerman et al 1992). A kit for measuring ET-1 was provided by Beijing Northern Bioengineering Institute.

mRNA expression by RT-PCR

The total RNA was extracted from 100 mg frozen LV tissue sample (n=5) and reversely transcribed into cDNA as described previously (Zhang et al 2005a). To ensure a fixed amount of initial mRNA in parallel with GAPDH, amplification was performed using the following oligonucleotides: sense, 5′-GCT GGG GCT CAC CTG AAG G-3′; antisense, 5′-GGA TGA CCT TGC CCA CAG CC-3′. Specific oligonucleotide primers for ryanodine receptor type 2 (RyR2), FKBP12.6, SERCA2a, phospholamban (PLB), iNOS, preproET-1, endothelin converting enzyme (ECE) and endothelin receptor A and B (ET_AR, ET_BR) were used and processed as described in previous work (Zhang et al 2005b; He et al 2006).

The amplification products were separated by agarose gel electrophoresis (1.7%), stained with ethidium-bromide and the bands were analysed by Labworks imaging acquisition and analysis software (Ultra-Violet Products, Cambridge, UK).

Protein expression by western blotting analysis

Left ventricular mass of 100 mg was homogenized in lysis buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, PMSF 100 mg mL^{-1}, aprotinin 1 mg mL^{-1}, 1% NP-40, 0.5% sodium deoxycholate). The lysate was analysed for protein content using a Bradford assay (Bio-Rad, CA). Equal amounts of protein were resolved under reducing conditions on 10% SDS-polyacrylamide gel for SERCA2a and 15% SDS-polyacrylamide gel for PLB and FKBP12.6. Immunoblotting was performed using antibody to SERCA2a, PLB and FKBP12.6 (Santa Cruz Biotechnology, Inc, CA) at a dilution of 1:100 (or 1:200) in non-fat milk–Tris buffer. The membrane was subsequently probed with a secondary anti-goat antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc, CA) at a dilution of 1:500 and detected with 0.1% 3,3′-diaminobenzidine–0.01% hydrogen peroxide. The bands were analysed by Labworks imaging acquisition and analysis software (Cambridge, UK).

Statistical analysis

Data were expressed as mean ± s.d. The one-way analysis of variance assay was performed and differences between two groups were compared by Student-Newman-Keuls test; survival rates were compared by a Chi square test. The results were considered significant at $P < 0.05$.

Results

Survival rate and cardiac weight index

Treatment of CPU0213 was initiated at 8 h after the diffused infection in the peritoneal cavity by CLP. The average survival time was 38.2 ± 3.3 h and survival rate was 9.1% at 72 h ($P < 0.01$) in untreated rats, compared with zero death in the sham operation group. The average survival time and survival rate were prolonged to 53.6 ± 4.0 h and 35.2%, respectively, by CPU0213 relative to the untreated model group (Figure 2A). Significant increase in the cardiac weight index (HW/BW and LVW/BW) by 14.6% ($P < 0.01$) and 21.4% ($P < 0.01$) was found in the septic AHF relative to the sham operation group, respectively, and regression by CPU0213 was significant by 47.4% ($P < 0.01$) and 81.1% ($P < 0.01$) compared with the untreated model group (Figure 2B, C). The body weight (g) of the sham, untreated and treated groups was not changed: 263.9 ± 11.5, 257.3 ± 12.7 and 260.9 ± 10.1 g, respectively.

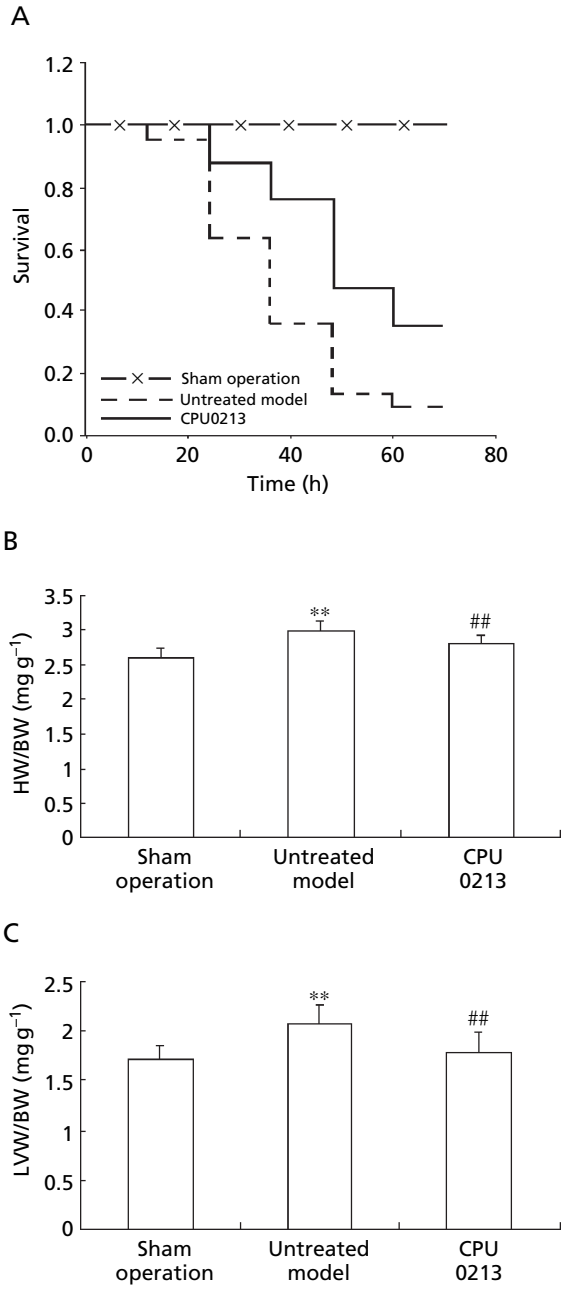

Figure 2 Improvement in the survival rate (A) and cardiac weight index (HW/BW (B) and LVW/BW (C)) of septic shock in rats by CPU0213 (30 mg kg^{-1}, s.c., every 12 h, for 3 days). Sham operation group, n = 15; untreated model group, n = 22; CPU 0213 group, n = 17. Data are shown as the mean ± s.d., n = 10. $^{**}P < 0.01$ vs sham operation group; $^{\#\#}P < 0.01$ vs the untreated model group. HW, heart weight; LVW, left ventricle weight; BW, body weight.

Effect on arterial pressure, heart rate and haemodynamics

Mean arterial pressure (MAP) in the untreated model group was markedly reduced by 36.9% ($P < 0.01$) relative to the sham operation group, and was restored partially by CPU0213 ($P < 0.01$). An increased heart rate by 30.7% was found in the untreated model group and was reversed by CPU0213 (Table 1).

In the untreated model group, there was a significant decrease in systolic function by 37.9% ($P < 0.01$) and 61.5% ($P < 0.01$) in LVSP and LV + dp/dtmax, and a compromised diastolic function by 396.4% ($P < 0.01$) and 42.0% ($P < 0.01$) in LVEDP and LV − dp/dtmin relative to the sham operation group, respectively. Administration of CPU0213 brought about

Table 1 Depression of cardiac function in AHF of septic shock rats was induced by CLP and improved by CPU0213 (30 mg kg^{-1}, s.c., every 12 h, for 3 days)

Group	MAP (kPa)	HR (beats/min)	LVSP (kPa)	LVEDP (kPa)	LV + dp/dtmax (kPa s^{-1})	LV–dp/dtmin (kPa s^{-1})
Sham operation	13.69 ± 1.20	347 ± 54	22.94 ± 1.38	0.55 ± 0.13	1313.40 ± 127.25	−900.01 ± 52.02
Untreated model	8.64 ± 1.03**	453 ± 31**	14.26 ± 1.48**	2.73 ± 0.43**	505.13 ± 75.45**	−521.61 ± 39.62**
CPU0213	12.0 ± 0.78##	400 ± 43##	20.22 ± 1.78##	1.36 ± 0.49##	887.88 ± 105.50##	−699.33 ± 55.77##

All data are expressed as the mean ± s.d., n = 10. MAP, mean arterial pressure; HR, heart rate. **$P < 0.01$ vs sham operation group; ##$P < 0.01$ vs the untreated model group.

a marked amelioration in changes of systolic and diastolic function, ($P < 0.01$) compared with the untreated rats (Table 1).

Effects on the Ca^{2+} handling system

In the LV tissue, mRNA expression levels of RyR2, FKBP12.6, SERCA2a and PLB were down-regulated significantly in the untreated model group, by 40.8%, 42.2%, 52.9% and 45.3% of the normal ($P < 0.01$), respectively. Abnormal mRNA expression of the Ca^{2+} handling system was prominent in AHF following septic shock. Application of CPU0213 8 h following peritoneal infection dramatically reversed down-regulated mRNA of the cardiac Ca^{2+} handling system relative to the untreated model group ($P < 0.05$ and $P < 0.01$, respectively; Figure 3A, B).

Significant changes of protein expression levels of FKBP12.6, SERCA2a and PLB were also observed in the LV tissue of the septic shock rats. In the untreated model group, protein abundance of FKBP12.6, SERCA2a and PLB was decreased by 53.0%, 50.8% and 56.6% in the LV tissue ($P < 0.01$), respectively, compared with the sham operation group. Treatment with CPU0213 effectively corrected the down-regulated protein abundance ($P < 0.05$ and $P < 0.01$) relative to the untreated model group, respectively (Figure 3C–E).

Effect on the ET signalling pathway

ET-1 concentrations of the LV increased significantly in the untreated model group by 55.4% ($P < 0.01$) relative to the sham operation group. Elevated ET-1 levels were reduced down to normal following administration of CPU0213 (Figure 4A).

In the untreated LV, all mRNA expressions of the preproET-1, ECE, ET_A and ET_B were up-regulated significantly, with an increment in abundance of mRNA by 93.9%, 82.4%, 103.5% and 78.4%, respectively ($P < 0.01$), relative to the sham operation group. Activation of the ET pathway was prominent in AHF following septic shock. CPU0213 dramatically reversed the up-regulation of the cardiac ET system ($P < 0.05$ and $P < 0.01$). Enhancement in the ET system was effectively blocked by endothelin receptor antagonist CPU0213 (Figure 4B, C).

Effect on the redox system

MDA production of the untreated LV tissue increased by 110.9% ($P < 0.01$); GSH-Px and SOD activity decreased by 51.7% and 28.3%, respectively ($P < 0.01$), relative to the sham operation group. This showed a state of oxidative stress in the myocardium of septic AHF, in association with an up-regulation of the ET system. CPU0213 significantly relieved oxidative stress by ameliorating the impaired redox system ($P < 0.01$) compared with the untreated model group (Table 2).

Effect on iNOS and NO

The iNOS activity and NO concentration of the untreated LV significantly increased by 287.0% and 228.0%, respectively ($P < 0.01$), relative to the sham operation group. An increased NO derived from the upregulated iNOS was significant and non-physiological. mRNA expression of iNOS in the untreated LV was significantly up-regulated by 113.3% ($P < 0.01$) compared with the sham operation group. Following CPU0213 treatment these effects were significantly suppressed ($P < 0.01$) relative to the untreated model group (Figure 5A–C).

Discussion

We have demonstrated the acute heart failure (AHF) model induced by CLP in rats in which an over-activated ET system and oxidative stress play important roles in the development of AHF. These changes lead to cardiac insufficiency associated with abnormality of the calcium handling system. The novel dual endothelin receptor antagonist CPU0213 relieves the above abnormalities in septic AHF.

Activated ET system

Acute cardiac insufficiency of septic shock in rats manifests as compromised cardiac performance, increased HR and high mortality in the untreated model group. The depression in systolic LVSP and LV + dp/dtmax, as well as diastolic LVEDP and LV–dp/dtmin, is attributed to myocardial damage by an excess of cytokines, such as ET-1, iNOS and ROS (Thiru et al 2000; Ishimaru et al 2001; Salvemini & Cuzzocrea 2002) in septic shock. ET-1 is produced in substantial amounts from the diseased myocardium and plays a critical role in the progression of AHF. In the study, increased ET levels in the LV tissue were found and served as a sensitive predictor for assessment of the severity of septic shock. Excessive levels of ET have been implicated in the

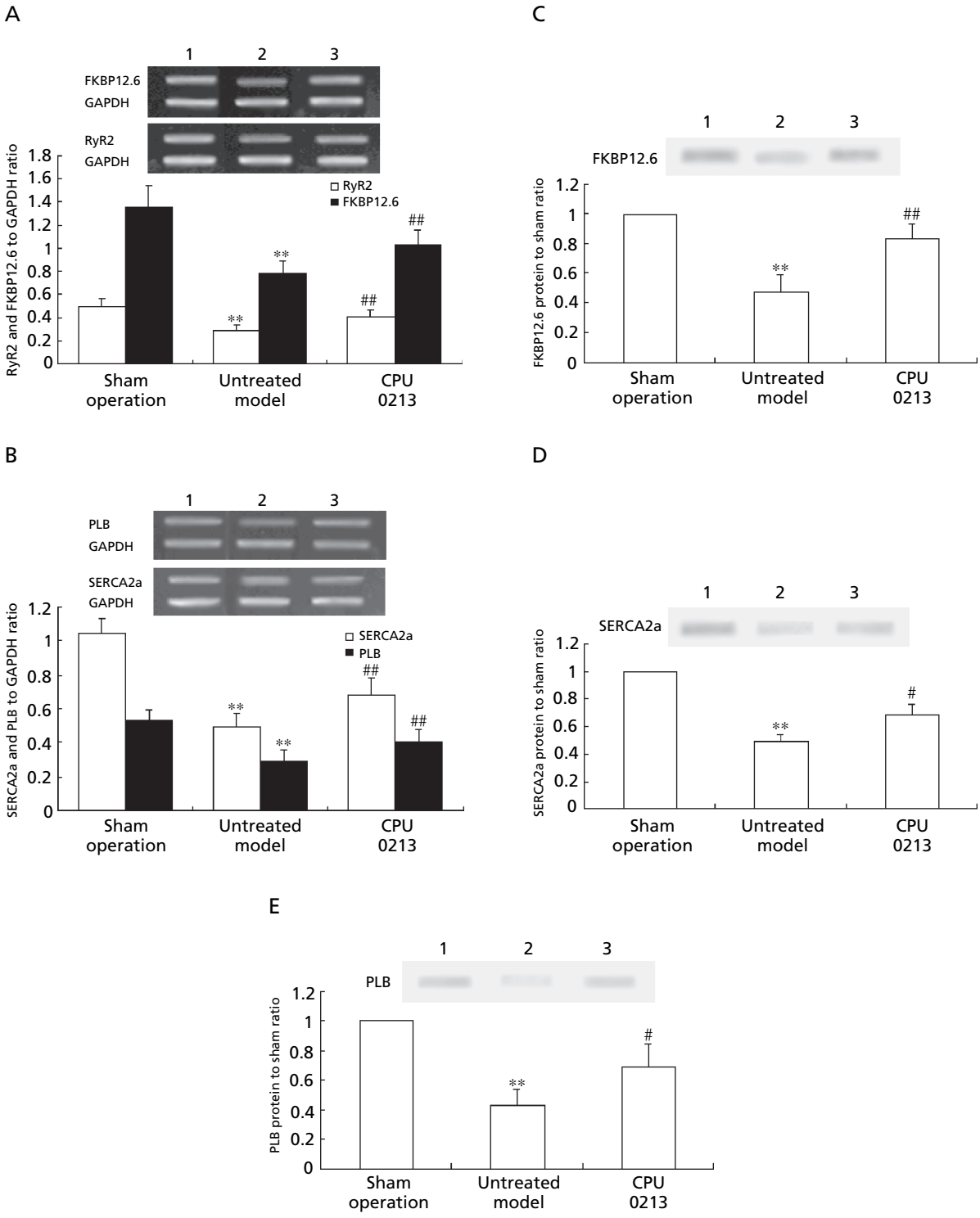

Figure 3 Change in mRNA and protein expression of Ca^{2+} handling system of SR in the LV tissue of the septic shock rats induced by CLP. These changes were eliminated by treatment with CPU0213. A. mRNA of RyR2 and FKBP12.6. B. mRNA of SERCA2a and PLB. C. The relative protein amount of FKBP12.6. D. The relative protein amount of SERCA2a. E. The relative protein amount of PLB. Lane 1, sham operation group; lane 2, untreated model group; lane 3, CPU0213 group. Data are shown as the mean ± s.d., n = 5. $^{**}P < 0.01$ vs sham operation group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the untreated model group

pathophysiology of renal vasoconstriction and sodium retention in animal models of CHF (Jerkic et al 2004), and participate in septic kidney (He et al 2006). An activated ET system in AHF of septic shock is revealed by not only an

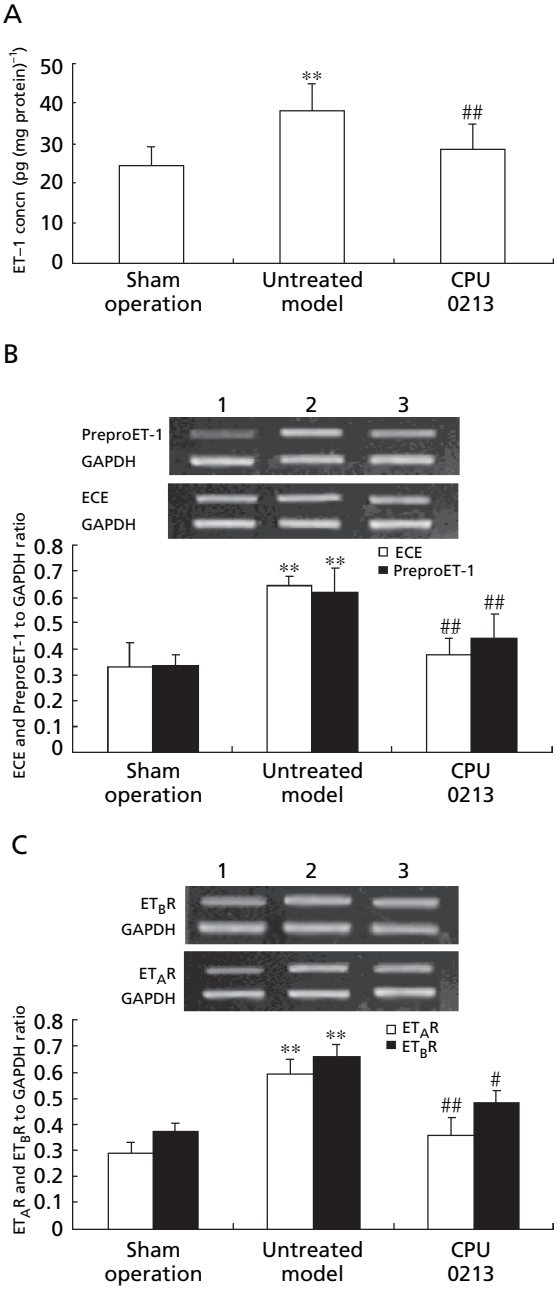

Figure 4 Elevated ET-1 and up-regulation of mRNA expression levels of ECE, preproET-1, ET_A and ET_B receptors in LV tissue of septic shock in rats induced by CLP and suppressed by treatment of CPU0213 (30 mg kg^{-1}, s.c., every 12 h, for 3 days). A. ET-1 concentrations (n = 10). B. mRNA of ECE and PreproET-1. C. mRNA of ET_A and ET_B receptors. Lane 1, sham operation group; lane 2, the untreated model group; lane 3, CPU0213 group. Data are shown as the mean ± s.d., n = 10 and 5. $^{**}P < 0.01$ vs sham operation group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the untreated model group.

increase in myocardial ET-1, up-regulation of mRNA of preproET-1 and ECE, but also up-regulation of both the ET_A and ET_B receptors. It is interesting to find that the involvement of an activated ET_B receptor is implicated in AHF of septic shock. Activation of ET_B receptors mediates constriction of the blood vessels in pathological conditions (Ohkita et al 2005; Wang et al 2006) and plays a role in vascular remodelling in grafted internal mammary artery (Sutherland et al 2006), and an inotropism on the myocardium can be mediated by the activity of ET_B receptors, which is implicated in cardiac insufficiency (Bras-Silva & Leite-Moreira 2005). In breast cancer, suppression of the activated ET_B receptors provides an additional benefit to paclitaxel (Rajeshkumar et al 2005). Changes in ET_A and ET_B receptors in AHF of septic shock coincide with over-expression of both ET_A and ET_B receptors in the failing rat heart (Kobayashi et al 1999). Thus, an inhibition of ET_B receptor is likely implicated in the benefit of CPU0213 to AHF in septic shock.

Table 2 Alteration of the redox system of the left ventricular myocardium in AHF of septic shock in rats and improvement by treating with CPU0213 (30 mg kg^{-1}, s.c., every 12 h, for 3 days)

Group	SOD (U (mg protein)$^{-1}$)	GSH-Px (U (mg protein)$^{-1}$)	MDA (nmol (mg protein)$^{-1}$)
Sham operation	92.65 ± 7.51	13.62 ± 3.26	9.42 ± 1.98
Untreated model	66.44 ± 5.70**	6.57 ± 1.65**	19.87 ± 5.02**
CPU0213	74.92 ± 6.01##	11.20 ± 1.96##	13.52 ± 2.75##

All data are expressed as the mean ± s.d., n = 10. $^{**}P < 0.01$ vs sham operation group; $^{\#\#}P < 0.01$ vs the untreated model group.

Activated oxidative stress and NOS system

Considerable evidence suggests that oxidant stress plays a major role in the progression of AHF (Demirbag et al 2005; Smith et al 2005). A relief in heart failure is accompanied with a decrease in oxidants in an affected myocardium (Wang et al 2004). When the endogenous antioxidant reserve is compromised, the myocardium predisposes to be damaged in an oxidative stress episode. The ROS are actual components linked with down-stream events of the ET signalling pathway (Li et al 2003) and an increase in ET release subsequently promotes an exaggerated ROS production (Kahler et al 2000). Blockade of the ET receptors by CPU0213 is followed by substantial inhibition of ROS production leading to a dramatic attenuation in the balance between oxidants and antioxidants of the redox system in the myocardium. The suppressive effect on oxidative stress by CPU0213 is in line with the beneficial effect of bosentan, which suppresses an episode of oxidative stress by ischaemia/reperfusion injury (Gupta et al 2005).

A copious amount of NO is produced from activation of iNOS activity that exacerbates inflammatory reactions in the myocardium by septic shock (Wanecek et al 2000). ET-1 and ROS contribute significantly to deleterious effects of endotoxin in producing vascular and cardiac dysfunction and depression (Kobayashi et al 1999). An increase in NO from iNOS promotes formation of peroxynitrite $ONOO^{\bullet -}$, which is toxic, to worsen cardiovascular dysfunction. These abnormalities are reversed by CPU0213.

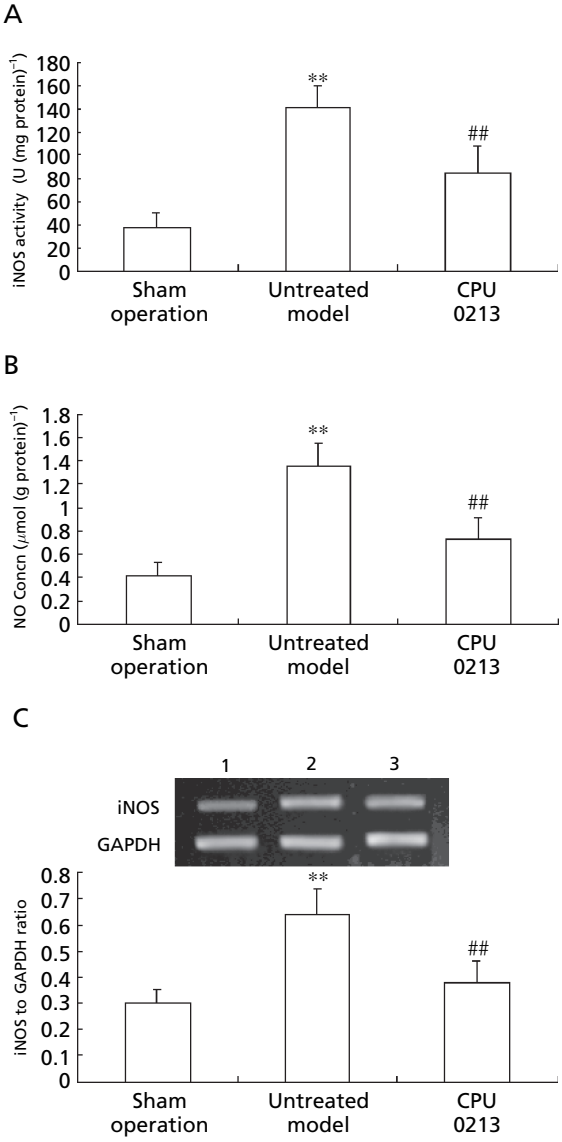

Figure 5 Elevated iNOS activity (A), NO concentration (B) and up-regulation of mRNA expression level of iNOS (C) in LV tissue of septic shock in rats induced by CLP and suppressed by treatment of CPU0213 (30 mg kg^{-1}, s.c., every 12 h, for 3 days). Lane 1, sham operation group; lane 2, the untreated model group; lane 3, CPU0213 group. Data are shown as the mean ± s.d., n = 10 and 5. $^{**}P < 0.01$ vs sham operation group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the untreated model group.

Dysfunction of the calcium handling system

A down-regulation of FKBP12.6 is an attractive molecular event in mechanisms underlying cardiac insufficiency (Marks 2003; Olson 2004) and serves as a target for new drug discovery (Yano et al 2003; Wehrens & Marks 2004; Guan et al 2006). In this study, a significant down-regulation of FKBP12.6 was observed in AHF of septic shock and likely indicates an unstable calcium release channel of RyR2. A depressed FKBP12.6 and SERCA2a of the intracellular calcium handling system are concomitantly involved to reflect an increased Ca^{2+} at the diastole in the affected myocardium of AHF following septic shock. Compromised cardiac performance of AHF stems from an elevated Ca^{2+} in diastole, by a calcium leak from an instable RyR2 and a less active SERCA2a. These can be subsequent to an over activity of the β-adrenergic receptors, and subsequently an over-phosphorylation of RyR2 and PLB mediated by PKA (Marks 2003; Olson 2004). On the other hand, we found in this study that down-regulation of the Ca^{2+} handling system is linked with up-regulation of mRNA levels of preproET-1, ECE, ET_A and ET_B receptors in AHF of septic shock, which may indicate that PKA phosphorylation is likely modulated by an activated ET signalling system. A link between the down-regulation of FKBP12.6 and SERCA2a and up-regulation of the ET system can be established by the fact that CPU0213 significantly benefits cardiac performance in septic AHF. An activated ET system likely mediates the abnormality of expression of the calcium handling system in the myocardium.

Conclusion

A down-regulation of FKBP12.6, SERCA2a and PLB in association with an up-regulation of preproET-1, ECE, ET_A and ET_B receptors, and iNOS contributes to AHF of septic shock. CPU0213, a novel dual endothelin receptor antagonist, is effective in eliminating these abnormalities. It suggests that an activation of both ET_A and ET_B receptors exerts an adverse role in the pathological progression of AHF.

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