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Inflammatory factors that contribute to upregulation of ERG and cardiac arrhythmias are suppressed by CPU86017, a class III antiarrhythmic agent

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Abstract

The aim of this study was to verify whether exaggerated arrhythmogenesis is attributed to inflammatory factors actively involving an excess of reactive oxygen species (ROS), transforming growth factor (TGF)-β and endothelin (ET). We hypothesized that CPU86017, derived from berberine, which possesses multi-channel blocking activity, could suppress inflammatory factors, resulting in inhibition of over-expression of ether-a-go-go (ERG) and an augmented incidence of ventricular fibrillation (VF) in ischaemia/reperfusion (I/R). Rats with cardiomyopathy (CMP) induced by thyroxine ($0.2\,mg^{-1}kg^{-1}$ s.c. daily for 10 days) were treated with propranolol ($10\,mg\,kg^{-1}$ p.o.) or CPU86017 ($80\,mg\,kg^{-1}$ p.o.) on days 6–10. On the 11th day, arrhythmogenesis of the CMP was evaluated by I/R. In the CMP control group, an increase in VF incidence was found with the I/R episode, accompanied by increased ROS, which manifested as an increased level of malondialdehyde and decreased activities of SOD, glutathione peroxidase and catalase in the myocardium. Levels of inducible nitric oxide synthase and TGF-β mRNA were increased in association with upregulation of preproET-1 and ET-converting enzyme. We found increased levels of ERG, which correlated well with arrhythmogenesis. Treatment with CPU86017 or propranolol reversed these changes. These experiments verified our hypothesis that the inflammatory factors ROS, iNOS, TGF-β and ET-1 are actively involved in upregulation of ERG and arrhythmogenesis. CPU86017 and propranolol reduced VF by suppressing these inflammatory factors in the myocardium.

Introduction

Unexplained syncope or sudden cardiac death (SCD) in a child or young adult, especially during physical exertion or emotional agitation, may correlate with an ion channelopathy such as long QT syndrome (LQTS), probably due to mutation of genes encoding the channels (Gao et al 2007; Koo et al 2007). LQTS is characterized by prolongation of action potential duration (APD) and is a risk for SCD attributed to torsades de pointes arrhythmias (Oliveira et al 1999; Wolbrette 2004). APD reflects the balance of total currents through the membrane during repolarization, and is sensitive to the rapid delayed rectifier outward potassium channel (IKr), which is mainly encoded by the *hERG* gene. In fact, IKr resulting from prolongation of APD is involved in both the anti-arrhythmic activity and pro-arrhythmic actions of drugs. Thus, the IKr is attractive for its important role in APD and arrhythmias (Tamargo et al 2004).

Loss-of-function mutations in the *hERG* gene account for a decreased IKr and prolongation of APD, contributing to LQTS (Curran et al 1995; Schwartz 2005). In recent years, a gain-of-function mutation of *hERG* has been found to facilitate K^+ efflux by enhancing IKr, resulting in shortening of the APD, known as short QT syndrome (SQTS) (Cerrone et al 2006; Witchel 2007). In addition, Taglialatela et al (1997) suggested that reactive oxygen species (ROS) can specifically increase the permeability of hERG K^+ channels, and suggested that an increase in ROS in the myocardium may predispose to syncope or SCD, possibly because of over-expression of the ether-a-go-go (ERG) protein. Emerging data suggested that the endothelin-1 (ET-1) pathway is likely to be implicated in cardiac arrhythmias (Horkay et al 2000) by modulating calcium-handling proteins in the sarcoplasmic reticulum (Feng et al 2007a). However, there is no direct evidence regarding the relevance of ROS to increased expression of ERG.

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Berberine, an ingredient of traditional Chinese medicine, has effects on inflammation (Kuo et al 2004), ion channels (Hua & Wang 1994; Xu et al 1997) and arrhythmias. However, its bioavailability is very low; thus, the derivative CPU86017 (7-(4-chlorbenzyl) -7, 8, 13, 13a-tetrahydroberberine chloride) was developed by chemical modification, improving both the bioavailability and calcium antagonism (Dai et al 2004). Based on accumulated data, it has been suggested that CPU86017 is a complex class III antiarrhythmic agent (Dai 2006).

Our previous studies showed that CPU86017 relieves damage to endothelial cells by suppressing oxidative stress and inflammatory factors (Du et al 2005), which may contribute to its potent activity in relieving cardiac arrhythmias (Dai 2006). Thus, we hypothesized that the antiarrhythmic activity of CPU86017 may be mediated by ROS scavenging and suppression of inflammatory factors. In this study, we investigated whether oxidant and inflammatory factors contribute to upregulation of the *ERG* gene (K^+ channelopathy) which correlates with ventricular fibrillation (VF) and whether these changes are suppressed by CPU86017.

Materials and Methods

Animals and surgical preparation

Adult male Sprague–Dawley rats (220–250 g) were used. The care and use of animals in this work was in accordance with Guidelines for the Care and Use of Laboratory Animals in Jiangsu Province, People's Republic of China; the experimental procedures were approved by the local Committee on Animal Care and Use.

The animals were divided randomly into four groups: control, cardiomyopathy (CMP) induced by thyroxine, and treatment of CMP rats with propranolol or CPU86017. CMP was induced by L-thyroxine ($0.2\,mg^{-1}kg^{-1}$ s.c. daily for 10 days). On day 6–10, animals were also given either propranolol ($10\,mg^{-1}kg^{-1}$ p.o.) or CPU86017 ($80\,mg^{-1}kg^{-1}$ p.o.). Animals in the control group received the same volume of vehicle (0.5% sodium carboxymethyl cellulose) p.o.

On the 11th day, rats were anaesthetized with urethane ($1.5\,gkg^{-1}$ i.p.) and the ischaemia/reperfusion (I/R) procedure was performed: coronary artery ligation of the left coronary artery was performed according to the method of Yu et al (1997), followed 10 min later by reperfusion. Occurrence of VF was identified by replacement of QRS complexes with irregular waves in the lead II electrocardiogram in association with a rapid drop in blood pressure. The heart was rapidly harvested and divided into the right and left ventricles (including the septum). The left ventricle (LV) and heart weight (HW) to body weight (BW) ratios were determined and compared among groups.

Biochemical parameters

Levels of myocardial malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-px), nitric oxide (NO), total nitric oxide synthase (tNOS), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) in the LV were determined using commercially available kits (Jiancheng Bio-engineering company, Nanjing, China). Protein was measured by the method of Lowry et al (1951).

Reverse transcription-polymerase chain reaction (RT-PCR)

Frozen tissue samples from the LV were homogenized, and total RNA was extracted using the acid–phenol–guanidinium–thiocyanate method (Bird 2005). We performed RT-PCR with standard methods, using 1 μg total RNA. PCR amplification was then performed with synthetic gene-specific primers, listed in Table 1. Amplification was performed using a PCR System Thermal Cycler (Eppendorf, Saxony, Germany) with parameters of 94°C for 30 s, then 40 s at different temperatures (Table 1) and 72°C for 1 min. The amplified PCR products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide and were expressed relative to the corresponding densities of the GAPDH bands from the same RNA sample.

Western blot analysis

LV muscle, frozen in liquid nitrogen, was homogenized and dissolved in five volumes of extraction buffer. Expression of ERG protein was determined by Western blot

Table 1 PCR primer sequences and annealing temperatures

Primer name	Primer	Size (bp)	Annealing temp (°C)	No. cycles
Inducible nitric oxide synthase	Sense: 5′-CTTCAGGTATGCGGTATTGG-3′ Antisense: 3′-CATGGTGAACACGTTCTTGG-5′	351	52	28
Prepro-endothelin 1	Sense: 5′-AGCAATAGCATCAAGGCATC-3′ Antisense: 3′-TCAGACACGAACACTCCCTA-5′	465	64	30
Endothelin-converting enzyme	Sense: 5′-CGTAGCGATAGTCTTAGCAC-3′ Antisense: 3′-GTGCCACACCAAAACTACAG-5′	515	54	30
Transforming growth factor-β1	Sense: 5′-AATACGTCAGACATTCGGGAAGCA-3′ Antisense: 3′-GTCAATGTACAGCTGCCGTACACA-5’	498	54	32
GAPDH (control)	Sense: 5′-GCTGGGGCTCACCTGAAGG –3′ Antisense: 3′-GGATGACCTTGCCCACAGCC –5′	343	55	34

according to our previous paper (Na et al 2007). After 15 min on ice, nuclei and cell debris were removed by centrifugation at 1000 *g* for 15 min at 4°C. The supernatants were then centrifuged at 10000 *g* for 40 min at 4°C, and the pellet was resuspended in the same buffer. Total cell lysates were mixed with SDS-PAGE sample buffer and boiled for 10 min at 100°C. The samples were subject to SDS-PAGE using the Laemmli buffer system (Gallagher 2006), and transferred to nitrocellulose membranes (Bio-Rad, Wien, Austria). Membranes were then blocked with 5% non-fat dry milk (NFDM) in Tris-buffered saline with 0.1% Tween 20 (TTBS). Primary rabbit-anti-ERG antibodies (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, USA) were diluted with 1% NFDM in TTBS and incubated with membranes overnight at 4°C. This was followed by three washes in TTBS and re-blocking of membranes with 1% NFDM in TTBS. Horseradish peroxidase-conjugated goat secondary antibody immunoglobulin G (1:1000, Santa Cruz Biotechnology) were diluted in 5% NFDM and applied to membranes at room temperature for an additional 1 h. After three washes, the blot incubated with antigen was detected with a 3,3′-diaminobenzidine kit (Wuha Boster Biological Technology, Wuhan, China). A linear relationship between blot density and protein load was observed when 20, 40, 60, 80 and 100 μg membrane protein were used per lane.

Statistical analysis

SigmaPlot 9.0 (SPSS Inc., Chicago, USA) was used to analyse the results. Data are presented as mean ± s.e. The paired Student's *t*-test was used for statistical comparison of mean values between two experimental groups, and one-way analysis of variance followed by Bonferroni's test was used to compare mean values between all experimental groups. A P value below 0.05 was considered to indicate statistical significance. GraphPad Prism V3.0 (GraphPad Software, San Diego, CA, USA) was used for analysis of electrophysiological data.

Results

Cardiac hypertrophy and VF

Rats with CMP induced by L-thyroxine exhibited cardiac hypertrophy, assessed by the HW/BW and LVW/BW ratios. The CMP group showed a significant increase in the HW/BW and LVW/BW ratios compared with the control group ($P < 0.01$, Table 2). The changes in HW/BW and LVW/BW were significantly reversed by intervention with propranolol or CPU86017.

An increased susceptibility to VF was evaluated by irregular QRS on reperfusion. In controls rats, the incidence of VF was very low with I/R, although frequent ventricular ectopic beats were found. In contrast, susceptibility to VF was significantly augmented in the CMP group compared with the control group (Figure 1). In the two treated groups, the incidence of VF was greatly reduced towards normal. However, ectopic ventricular beats were still found. The data showed that life-threatening arrhythmias were significantly eliminated compared with untreated CMP ($P < 0.05$). Thus, intervention with propranolol or CPU86017 can notably improve both myocardial hypertrophy and susceptibility to VF.

Oxidative stress

A status of oxidative stress was predominant in the CMP myocardium. This is attributed to an increase in MDA and a reduction in SOD and GSH-px activity compared with controls (Table 2). Both propranolol and CPU86017 suppressed these changes compared with the untreated group ($P < 0.05$). Oxidative reactions in the myocardium were prevented by drug treatment, in conjunction with regression of ventricular hypertrophy and arrhythmogenesis.

NO and NOS activity

The level of NO and activities of tNOS and eNOS in the LV were decreased significantly after I/R, as shown in Table 2.

Table 2 Effects of propranolol and CPU86017 on weight ratios, and the state of oxidative stress and the nitric oxide system of the left ventricle in rats with thyroxine-induced cardiomyopathy (CMP)

	Control	CMP	CMP + propranolol	CMP + CPU86017
LV/BW (%)	0.21 ± 0.01	0.31 ± 0.02**	0.27 ± 0.01†	0.26 ± 0.01†
HW/BW (%)	0.31 ± 0.01	0.46 ± 0.02**	0.39 ± 0.02†	0.4 ± 0.01†
SOD	156.9 ± 5.9	92.2 ± 4.2**	138.6 ± 5.1†	123.6 ± 6.7
MDA	4.91 ± 0.52	8.52 ± 0.81**	4.85 ± 0.34‡	5.49 ± 0.48†
GSH-px	81.8 ± 8.5	45.9 ± 5.1**	79.6 ± 5.7‡	81.3 ± 4.1‡
NO	1.88 ± 0.1	0.59 ± 0.04**	1.09 ± 0.05‡	0.88 ± 0.09†
TNOS	0.72 ± 0.06	0.42 ± 0.04**	0.72 ± 0.06‡	0.64 ± 0.05†
eNOS	0.59 ± 0.06	0.23 ± 0.02**	0.55 ± 0.05‡	0.46 ± 0.03†
iNOS	0.13 ± 0.01	0.19 ± 0.03*	0.14 ± 0.02†	0.15 ± 0.02†

LV/BW left ventricle to body weight ratio; HW/BW, heart weight to body weight ratio; MDA, malondialdehyde; GSH-px, glutathione peroxidase; NO, nitric oxide; t/e/i NOS, total/endothelial/inducible nitric oxide synthase.
Data are means ± s.e. (n = 10).
*$P < 0.05$, **$P < 0.01$ vs control; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ vs CMP.

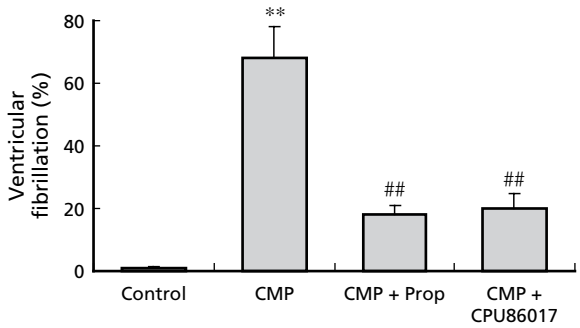

Figure 1 Susceptibility to ventricular fibrillation was increased in rats with thyroxine-induced cardiomyopathy (CMP) and decreased after intervention with propranolol (Prop) or CPU86017. Data are mean ± s.e. (n = 10). $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs CMP.

This suggested that the normal and physiological effect of NO was compromised, resulting from decreased activity of tNOS and eNOS in cardiomyopathy due to high doses of thyroxine. Conversely, the activity of iNOS was increased in the arrhythmogenic rat heart (Table 2, Figure 2C). These changes were dramatically reversed by either propranolol or CPU86017 ($P < 0.05$). These results indicate that function of endothelial cells was impaired, probably correlating to an increased pro-inflammatory activity of iNOS, and was normalized by propranolol or CPU86017.

Expression of preproET-1, ECE and TGF-β mRNA

It was interesting to see whether the expression of mRNA for preproET-1 and ET-converting enzyme (ECE) were changed during arrhythmogenesis in CMP. We found that these were individually upregulated, indicating activation of the ET system in the myocardium (Figure 2A, B). Treatment with propranolol or CPU86017 suppressed the abnormalities. Meanwhile, transforming growth factor (TGF)-β, an inflammatory factor, showed the greatest upregulation among these relative to the control (Figure 2D), and this was reversed by propranolol and CPU86017.

Over-expression of ERG protein

We have shown that increased incidence of VF in CMP correlates to changes in the expression of IKr channel (Dai & Yu 2005; Dai 2006). We therefore examined expression of the *ERG* gene in the CMP myocardium. An increase in ERG protein level was found in the untreated CMP group compared with the controls. Propranolol and CPU86017 significantly reversed the over-expression of the voltage-dependent delayed outward potassium channel in the myocardium ($P < 0.01$, Figure 3).

Discussion

The present study investigated the increased production of inflammatory factors in the myocardium correlating to increased incidence of VF found in rats with thyroxine-induced CMP. An increased susceptibility to VF was associated with a status of oxidative stress, which is expressed by an imbalance between cellular production of MDA and antioxidant defence molecules SOD and GSH, consistent with enhancement of ROS production under inflammatory conditions (Geronikaki & Gavalas 2006). Endogenous NO production was reduced, resulting from compromised eNOS activity. A reduced NO level is likely to exert an adverse effect on the normal function and structure of the heart. The production of NO from the endothelium is potently suppressed by an excess of ET-1, which is produced by an upregulation of the ET pathway (upregulated prepro-ET1 and ECE). The ET pathway exerts a number of biological effects that promote proliferation of the myocardium and that may change ion channels in the heart. Thus, antagonism of the activated ET system may be beneficial in suppressing cardiac arrhythmias (Mohácsi et al 2004; Feng et al 2007b). In the present study, activation of the ET pathway in association with a compromised NO system is likely to be implicated in the pathology of arrhythmogenesis in CMP. Myocardial iNOS activity, as we found in the present study, was increased, together with decreased eNOS activity in CMP. This is in line with our previous data (Du et al 2005). In addition, impairment of the endothelium was exacerbated by inflammatory factors contributing to predisposition of CMP hearts to VF on I/R.

TGF-β is an important modulator of myocardial remodelling. Thus, upregulation of TGF-β may alter signalling pathways, leading to ventricular hypertrophy. Cardiac arrhythmia has been found to correlate with activation of TGF-β, as found in atrial fibrillation (Khan & Sheppard 2006). This combines with increases in iNOS, ROS and ET-1 to exacerbate pro-inflammatory reactions, resulting in an increased susceptibility to cardiac arrhythmias. We conclude that the pro-inflammatory factors that result from oxidative stress, such as iNOS, ROS and ET-1, make the myocardium more susceptible to severe cardiac arrhythmias.

CPU86017 has similar anti-inflammatory activity to berberine (Kuo et al 2004). It is a potent suppressor of oxidative stress in-vivo (Dai 2006) and in-vitro (Hao et al 2005) and of the activated ET pathway (Zhang et al 2005). CPU86017 suppresses VF by normalizing an increase in inflammatory factors, which include an excess of ROS, activation of the ET pathway, enhanced iNOS activity and expression, and upregulation of TGF-β expression. We also found that propranolol, a β-receptor blocker, decreases susceptibility to VF, relating to its suppression of antioxidant activity (Anderson et al 1996) and other pro-inflammatory factors that lead to abnormal expression of ERG protein in the myocardium. This is consistent with previous reports that propranolol can be used to treat LQTS (Bar-Cohen & Silka 2006; Hobbs et al 2006).

We know that 90% of LQTS caused by genetic defects are attributed to loss-of-function mutations in the IKr genes *KCNQ1* (LQT1) and *KCNH2* (LQT2), predisposing the heart to life-threatening arrhythmias due to prolongation of APD/QTc. In contrast, inherited defects attributed to a gain-of-function mutation of ERG (*KCNH2*) have been found to augment the IKr current, which manifests as SQTS, associated with a high risk of SCD. Thus, the cardiac IKr needs to be kept within the normal range; both increases or decreases in IKr are associated with arrhythmogenesis. Interestingly, in

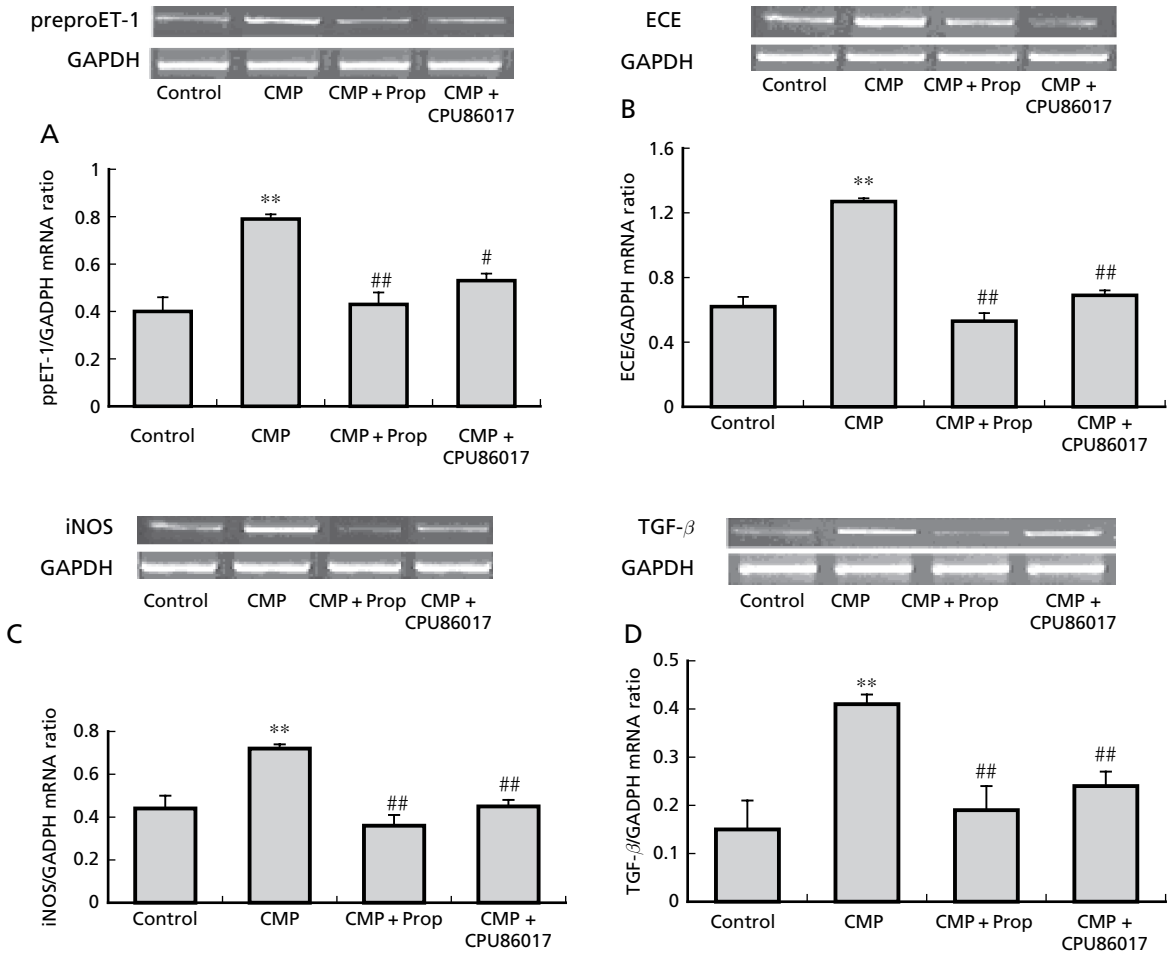

Figure 2 Upregulation of mRNA expression of: (A) prepro-endothelin 1 (ppET-1), (B) endothelin-converting enzyme (ECE), (C) inducible nitric oxide synthase (iNOS) and (d) transforming growth factor (TGF)-β in the left ventricle were increased significantly by ischaemia–reperfusion injury in rats with cardiomyopathy (CMP); these increases were suppressed by treatment with propranolol (Prop) or CPU86017. Data are mean ± s.e. (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs CMP.

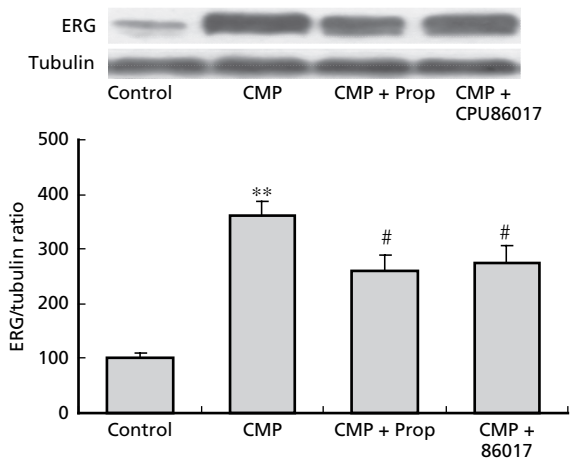

Figure 3 Over-expression of ERG protein in rats with cardiomyopathy (CMP) was associated with an increased incidence of ventricular fibrillation on ischaemia/reperfusion injury, and was normalized by either propranolol (Prop) or CPU86017. Data are mean ± s.e. (n = 4) $^{**}P < 0.01$ vs control; $^{\#}P < 0.05$ vs CMP.

the present study an upregulation of ERG protein was associated with an augmented IKr current in rats with CMP. In fact, we have previously reported that an enhanced IKr is found in isolated myocytes from rats with thyroxine-induced CMP (Zhang et al 2000). This may provide an explanation for exaggerated cardiac arrhythmias in CMP: the increased IKr current facilitates the repolarization process and leads to shortening of APD, which underlies an increased risk of arrhythmogenesis (Cerrone et al 2006). Our findings in the present experiment are in keeping with findings relating to gain-of-function mutations in *KCNH2* in SQTS (Giustetto et al 2006). This is the first time that increased expression of ERG protein has been reported in association with exacerbated arrhythmogenesis in CMP. Thus, increased levels of ERG protein could provide an alternative target for understanding the molecular events that underlie arrhythmogenesis and for evaluating the efficacy of antiarrhythmic agents (Taglialatela et al 1998; Witchel 2007). It is interesting to discover that inflammatory factors play a role in the upregulation of *ERG* to form K^+ channelopathy in the diseased myocardium associated with the majority of cases of SCD. These co-contribute to an increased susceptibility to VF on

stress (mental, physical or oxidative stress) such as with the reperfusion episode of I/R.

A protein FKBP12.7 (calstabin 2) stabilizes Ca^{2+} release from ryanodine receptor type 2 (RyR_2) during diastole, and calcium leak leading to cardiac arrhythmias can be attributed to unstable RyR_2, resulting from downregulation (dissociation) of FKBP12.6 (Lehnart et al 2006; Wehrens 2007). Some pro-inflammatory factors participate in downregulation of FKBP12.6, leading to an increased incidence of VF in CMP, and reversal of FKBP12.6 by suppressing ET-1, nuclear factor κB, tumour necrosis factor α or iNOS reduces the increased VF (Xia et al 2006). CPU86017 has similar anti-inflammatory activity to berberine (Kuo et al 2004) and normalizes over-expression of ERG by suppressing inflammatory factors, as demonstrated in the present study. Secondly, CPU86017 blocks both calcium and potassium currents (Dai 2006), which also contribute to its antiarrhythmic activity.

Conclusion

In this study we found that marked inflammatory reactions in the myocardium of rats with thyroxine-induced CMP made substantial contributions to the increased incidence of VF. An increase in the expression of ERG protein results in abnormalities in the repolarization process, which leads to an increased susceptibility to VF. Upregulation of the ERG protein and pro-inflammatory factors are important biomarkers that indicate predisposition of an affected heart to severe arrhythmias. CPU86017 has potent anti-arrhythmic activity, exerted at least in part by suppressing the augmented expression of ERG protein and pro-inflammatory factors.

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