Inhibiting Mitochondrial Na⁺/Ca²⁺ Exchange Prevents Sudden Death in a Guinea Pig Model of Heart Failure

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ABSTRACT

**Rationale:** In cardiomyocytes from failing hearts, insufficient mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{m}\)]\(_{m}\)) accumulation secondary to cytoplasmic Na\(^{+}\) overload decreases NAD(P)H/NAD(P)\(^{+}\) redox potential and increases oxidative stress when workload increases. These effects are abolished by enhancing [Ca\(^{2+}\)\(_{m}\)] with acute treatment with CGP-37157 (CGP), an inhibitor of the mitochondrial Na\(^{+}/Ca^{2+}\) exchanger.

**Objective:** To determine if chronic CGP treatment mitigates contractile dysfunction and arrhythmias in an animal model of heart failure (HF) and sudden cardiac death (SCD).

**Methods and Results:** Here, we describe a novel guinea-pig HF/SCD model employing aortic constriction combined with daily \(\beta\)-adrenergic receptor stimulation (ACi) and show that chronic CGP treatment (ACi+CGP) attenuates cardiac hypertrophic remodeling, pulmonary edema, and interstitial fibrosis and prevents cardiac dysfunction and SCD. In the ACi group 4 weeks after pressure-overload, fractional shortening and the rate of left ventricular pressure development decreased by 36% and 32%, respectively, compared to sham-operated controls; in contrast, cardiac function was completely preserved in the ACi+CGP group. CGP treatment also significantly reduced the incidence of premature ventricular beats and prevented fatal episodes of ventricular fibrillation, but did not prevent QT prolongation. Without CGP treatment, mortality was 61% in the ACi group within 4 weeks of aortic constriction, while the death rate in the ACi+CGP group was not different from sham-operated animals.

**Conclusions:** The findings demonstrate the critical role played by altered mitochondrial Ca\(^{2+}\) dynamics in the development of HF and HF-associated SCD; moreover, they reveal a novel strategy for treating SCD and cardiac decompensation in HF.

**Keywords:** mitochondria, Na\(^{+}/Ca^{2+}\) exchange, metabolism, NADH, reactive oxygen species, oxidative phosphorylation, Ca\(^{2+}\) regulation, CGP-37157, calcium regulation, oxidative stress.

**Nonstandard Abbreviations and Acronyms.**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>HF</td>
<td>heart failure</td>
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<tr>
<td>SCD</td>
<td>sudden cardiac death</td>
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<td>ICD</td>
<td>implantable cardiac defibrillator</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
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<tr>
<td>GSH</td>
<td>reduced glutathione</td>
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<tr>
<td>Trx(SH)2</td>
<td>reduced thioredoxin</td>
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<tr>
<td>[Na(^{+})](_{c})</td>
<td>cytoplasmic Na(^{+})</td>
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<tr>
<td>[Ca(^{2+})(_{m})]</td>
<td>mitochondrial Ca(^{2+})</td>
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<tr>
<td>MCU</td>
<td>mitochondrial Ca(^{2+}) uniporter</td>
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<tr>
<td>mNCE</td>
<td>mitochondrial Na(^{+}/Ca^{2+}) exchanger</td>
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<tr>
<td>CGP</td>
<td>CGP-37157 (7-Chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one)</td>
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<tr>
<td>AC</td>
<td>ascending aortic constriction</td>
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<tr>
<td>FS</td>
<td>fractional shortening</td>
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<tr>
<td>LVH</td>
<td>left ventricular hypertrophy</td>
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<tr>
<td>CM-DCF</td>
<td>5-(and -6)-chloromethyl-2(^{7})-dichlorodihydrofluorescein</td>
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<tr>
<td>LVIDd</td>
<td>diastolic LV internal dimension</td>
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<tr>
<td>HR</td>
<td>heart rate</td>
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<tr>
<td>PVB</td>
<td>premature ventricular beat</td>
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<tr>
<td>HRV</td>
<td>HR variability</td>
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<tr>
<td>SDRR</td>
<td>standard deviation of RR interval</td>
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rMSSD  root mean square of successive difference
SD1     short term dispersion
SD2     long term dispersion

INTRODUCTION

The clinical syndrome of heart failure (HF), which can occur after acute myocardial ischemic injury or following longstanding chronic cardiovascular disease, is a progressive disease often involving a phase of compensatory hypertrophy followed by a transition to impaired contractility (decompensation) with systolic and/or diastolic dysfunction. While mortality in the later stages of the disease is most often attributed to impaired pump function, sudden cardiac death (SCD) is the leading cause of mortality in patients with mild to moderate HF, accounting for 35-50% of deaths\(^1,2\), prompting an increase in the use of implantable cardiac defibrillators (ICDs) as prophylactic devices\(^3\). The mechanisms underlying SCD, and the transition from compensated to decompensated HF, are incompletely understood, but neurohormonal abnormalities, ion channel/transporter changes, Ca\(^{2+}\) handling alterations, the activation of intra- and extracellular signaling pathways, and oxidative stress, have all been implicated in the pathophysiology\(^4\).

A limitation of energy production and/or substrate utilization is also thought to contribute to HF\(^5-7\) and several studies have reported HF-associated defects in mitochondrial function\(^8-10\). Mitochondria are not only the predominant source of ATP, upon which normal cardiac function depends, but also play a critical role in other biological processes, such as redox regulation, intermediary metabolism, cell signaling, and ion homeostasis. In the context of HF, the most detrimental consequences of mitochondrial dysfunction, either as the primary or as a secondary factor in the development of HF, are impaired bioenergetics and increased oxidative stress. Reactive oxygen species (ROS) have complex effects on the development of HF, playing a key role in cardiac remodeling by activating multiple signaling pathways involved in hypertrophic growth of cardiomyocytes, apoptosis, and proliferation of fibroblasts\(^11\). Increased oxidative stress also impairs cardiac function directly by modifying the proteins involved in excitation-contraction coupling\(^12,13\).

Targeting mitochondria to improve bioenergetics and to maintain redox balance is emerging as a novel therapeutic strategy for HF\(^14-16\).

Upstream of oxidative stress, recent evidence has suggested that altered Na\(^+\) and Ca\(^{2+}\) gradients across the mitochondrial inner membrane may be a proximal factor in the imbalance of energy supply and demand\(^17,19\), as well as ROS balance\(^19\), in failing heart cells. The pathway of contractile and electrical dysfunction involves a vicious cycle whereby increased cytoplasmic Na\(^+\) ([Na\(^+\)]\(_c\)), along with impaired SR Ca\(^{2+}\) release, leads to blunted Ca\(^{2+}\) signaling to the mitochondria during increased work\(^17-20\). Consequently, the failure to stimulate Ca\(^{2+}\)-dependent dehydrogenases of Krebs cycle\(^21-25\) results in the net oxidation of the matrix NADH pool. The decreased NADH/NAD\(^+\) redox potential, on the one hand, results in a deficiency of reducing equivalents that drive oxidative phosphorylation (OXPHOS) and ATP production, and, on the other hand, compromises the ability to scavenge ROS, owing to the interdependence of the NADH and NADPH redox pools\(^20\) (see schema in Online Figure I). Mitochondrial NADPH is crucial because it provides the reducing power to maintain the reduced glutathione (GSH), thioredoxin (Trx(SH)\(_2\)) and glutaredoxin pools, all of which are vital for ROS scavenging and the prevention of protein thiol oxidative damage\(^26\).

Mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_m\)) is determined by the balance of influx through the mitochondrial Ca\(^{2+}\) uniporter (MCU) and efflux through the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger (mNCE); hence, a change in either of these pathways can disrupt mitochondrial Ca\(^{2+}\) dynamics. The Km of mNCE for Na\(^+\) is ~5-10 mmol/L, falling within the range of [Na\(^+\)]\(_c\) levels in cardiomyocytes\(^27\). Therefore, the elevated [Na\(^+\)]\(_c\) consistently observed in HF can significantly accelerate the [Ca\(^{2+}\)]\(_m\) efflux rate. Studies of isolated heart
mitochondria have shown that an increase of extramitochondrial Na⁺ leads to decreased [Ca²⁺]m and compromises mitochondrial energetics, including oxidation of the NADH pool, decreased OXPHOS rate and ATP levels. Similarly, elevated [Na⁺], in isolated myocytes from failing guinea pig hearts (or ouabain-treated myocytes) causes insufficient [Ca²⁺]m accumulation during increased work, net oxidation of NAD(P)H, and a massive increase of intracellular ROS, ultimately resulting in abnormal Ca²⁺ regulation and arrhythmias. The adverse effects of high [Na⁺] are abolished by CGP-37157 (CGP), an inhibitor of mNCE.

In the present study, we examine whether in vivo treatment with CGP prevents the development of HF and SCD using a novel guinea pig model of HF induced by left ventricular pressure overload combined with a daily β-adrenergic challenge. We show that partial inhibition of mNCE attenuates cardiac remodeling, preserves cardiac contractile function, and protects against HF-associated arrhythmias and SCD, significantly improving overall survival. The results highlight mNCE as a novel target for HF treatment.

METHODS
Methods are described in detail in the Online Supplement.

RESULTS

Daily β-adrenergic challenge accelerates the transition from LV hypertrophy to HF.

A guinea pig HF model was developed by combining ascending aortic constriction (AC) with daily administration of the β-adrenergic agonist isoproterenol via i.p. injection (ACi). The rationale was to expose the pressure-overloaded heart to a period of increased work each day to simulate β-adrenergic stress. The isoproterenol dose was chosen so that it would have minimal effects on cardiac hypertrophic remodeling in the absence of AC. There was no evidence of cardiac hypertrophic remodeling in sham-operated animals exposed to the 4-week isoproterenol challenge (SHAMI) as compared with the sham-operated, untreated group (SHAM). However, in the presence of AC, the same isoproterenol treatment significantly exacerbated the decline in cardiac fractional shortening (FS) evoked by AC alone (Online Figure II). In ACi, FS decreased from 46.5±1.0% at week 0 (pre-bandung) to 30.5±1.5% at week 4, whereas with AC alone, FS was not significantly decreased by week 4 (44.2±1.0% at week 0 versus 43.4±2.2% at week 4). Significant cardiac dysfunction in the AC group was observed only after 6 weeks of pressure overload (FS 38.4±1.2%), while at week 8, FS decreased to 29.3±3.3%, which was similar to the functional decline evident at week 4 in ACi (Online Figure II). Thus, the major effect of the daily isoproterenol challenge was to accelerate the transition from LV hypertrophy (LVH) to decompensated HF.

Inhibition of mNCE restores mitochondrial Ca²⁺ accumulation and prevents energetic mismatch and oxidative stress in myocytes from ACi hearts during a rapid increase in work.

Impaired mitochondrial Ca²⁺ signaling secondary to cytoplasmic Na⁺ overload is a major cause of energy/redox imbalance in myocytes from pressure-overload induced failing hearts and interventions that raise [Ca²⁺]m above a required threshold prevent cellular ROS overload. Therefore, we first examined whether the same defect was present in the ACi model and if acute CGP treatment could mitigate the problem in vitro. [Na⁺] overload was also observed in the ACi model. [Na⁺] in cardiomyocytes isolated from ACi heart was increased by 208% compared to that in myocytes from SHAMI hearts (4.8±1.2 mmol/L in Shami vs. 14.8±1.1 mmol/L in ACi) (Online Figure III). [Ca²⁺]m dynamics, monitored with MityCam...
fluorescence, showed that mitochondria take up Ca\(^{2+}\) on a beat-to-beat basis (Fig. 1A). Upon stimulation (0.1 Hz), \([\text{Ca}^{2+}]_m\) increased abruptly followed by decay during diastole. An increase of work from 0.1Hz to 1Hz stimulation resulted in a further increase in MityCam signal (1-F/F0) (Fig. 1A). Measurement of peak MityCam signal indicated that accumulation of \([\text{Ca}^{2+}]_m\) during increased work was significantly less in myocytes from ACi hearts than SHAMi hearts, whereas 1µmol/L CGP treatment fully restored \([\text{Ca}^{2+}]_m\) in the ACi myocytes (Fig. 1 A and B). Isolated cardiomyocytes were challenged with a rapid increase in work from the resting state to 4 Hz stimulation in the presence of 100 nmol/L isoproterenol. NAD(P)H autofluorescence was strongly oxidized during 4Hz-stimulation in ACi cardiomyocytes (Fig. 1A). NAD(P)H decreased from 67%±4.4 reduced at rest to 33%±5.7 at the end of the stimulation period, while in SHAMi myocytes subjected to the same protocol, NAD(P)H levels were well maintained (67%±5.1 reduced at pre-stimulation vs. 66%±4.8 at the end of stimulation). Notably, the oxidation of NAD(P)H in the ACi myocytes was prevented by treatment with 1µmol/L CGP-37157 (68%±5.5 reduced at pre-stimulation vs. 64%±9.1 at the end of stimulation) (Fig. 1A). CGP had no effect on the sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchange current at concentrations up to 10 \(\mu\)M (Online Figure IV A), confirming its specificity for the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger.

The oxidation of the NAD(P)H pool during increased work in ACi cells was associated with increased oxidative stress. ROS accumulation was monitored with the H\(_2\)O\(_2\)-sensitive fluorescent probe 5-(and -6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCF). CM-DCF oxidation rate in SHAMi myocytes was low, with no significant change induced by the increased work (Fig. 1B). In contrast, ROS accumulation in ACi cells was markedly increased upon stimulation (Fig. 1B). During stimulation, the CM-DCF oxidation rate in ACi cells was ~10-fold higher than that in SHAMi cells. The enhanced intracellular ROS accumulation rate in ACi cells was abolished by CGP-37157 treatment (Fig. 1B).

**Effects of chronic CGP-37157 treatment on ACi-induced cardiac remodeling and functional deterioration.**

Significant cardiac hypertrophy, pulmonary congestion, and interstitial fibrosis were present in the ACi group by the end of week 4. HW/TL was 53% higher in ACi (0.75±0.04 g/cm) versus SHAM (0.49±0.02 g/cm) and LW/TL was 66% higher in ACi (5.69±0.43 g/cm) versus SHAM (3.41±0.08 g/cm)(Fig. 2A). Histological analysis showed that interstitial fibrosis in ACi hearts was 12.5-fold higher than in SHAM (Fig. 2B). Isoproterenol challenge in the absence of pressure overload did not significantly increase fibrosis, although there was a trend towards more fibrosis in the SHAMi group relative to the SHAM controls (percent fibrotic tissue area was 0.53±0.2 in SHAM versus 1.1±0.5 in SHAMi, \(p=0.052\)) after 4-weeks of injections (Fig. 2B). CGP treatment protected against HF-associated cardiac remodeling: the ACi+CGP group developed less cardiac hypertrophy (HW/TL: 0.64±0.04 g/cm, \(p<0.05\) versus ACi) and pulmonary congestion (LW/TL: 4.04±0.22 g/cm, \(p<0.001\) versus ACi) at 4 weeks compared to the ACi group (Fig. 2A). CGP treatment also attenuated interstitial fibrosis, which was decreased by 50% in ACi+CGP group compared to that in ACi group (Fig. 2B).

Pressure overload with daily \(\beta\)-adrenergic challenge induced LVH and progressive cardiac dilation and impaired contractility within 4 weeks of surgery (Fig. 2C). Compared to SHAM, FS in the ACi group was decreased by 36% (28.9±1.6% in ACi vs. 44.9±0.9% in SHAM, \(p<0.001\)) and diastolic LV internal dimension (LVIDd) was increased by 51% (1.07±0.04 cm in ACi vs. 0.71±0.01 cm in SHAM, \(p<0.001\)) (Fig. 2C). Contractile impairment was also confirmed by in vivo hemodynamic studies: +dP/dt normalized to pressure (+dP/dt ip) in the ACi group was decreased by 32% compared to that of SHAM controls (73.5±4.0 in SHAM vs. 49.9±2.1 in ACi, \(p<0.001\)) (Fig. 2D). In contrast, animals treated with CGP displayed well-maintained cardiac function without LV dilation. No significant differences in FS, LVIDd, or +dP/dt ip between the SHAM and ACi+CGP groups were observed. \(\beta\)-adrenergic treatment alone (SHAMi vs. SHAM) had no effect on cardiac hemodynamic parameters.
Heart rate (HR) responses to the daily isoproterenol injection were recorded in a subset (3-6 guinea pigs) of animals from each experimental group by means of implanted biopotential telemetric transmitters. Baseline and maximal HR, determined by analysis of ECG acquired during and after isoproterenol injections, were compared at 1 and 4 weeks post-surgery (Online Figure V). Baseline HR was unaffected by chronic β-adrenergic treatment in the absence of pressure overload (SHAMi versus SHAM); however, the ACi group had higher baseline HR at weeks 1 and 4 after pressure overload compared to SHAMi controls (Online Figure V). Chronic treatment with CGP-37157 (ACi + CGP), administered continuously at dose of 0.015mg/kg/hour by means of an implanted osmotic pump, attenuated, but did not completely eliminate, the increase of baseline HR at week 1 but abolished it at week 4 (baseline HR at week 4: 218.8±9.0 bpm in SHAMi vs. 206.2±7.5 bpm in ACi+CGP). Following injection of isoproterenol, HR rapidly increased to a maximum of ~390-400 bpm in all groups and then slowly declined back to the baseline rate in 4-5 hours (Online Figure V). The peaks and kinetics of the normalized chronotropic response to isoproterenol were not significantly different between the experimental groups.

Antiarrhythmic effects of CGP-37157.

Telemetry studies revealed that animals in the ACi HF group had a high incidence of premature ventricular beats (PVB) (Fig. 3). PVB frequency was significantly decreased by CGP treatment. To evaluate the antiarrhythmic effects of CGP, PVB were counted in a 2 hour-period at baseline and a 4 hour-period following isoproterenol injection at weeks 1 (Fig. 3A) and 4 (Fig. 3B) after surgery. At baseline, occasional PVB were detected in all groups, but the incidence of PVB was very low in SHAM and SHAMi groups. Moreover, the rates did not change from week 1 to week 4 (week1: 3.3±2.0; week 4: 4.0±2.5 counts/hour). In contrast, at baseline, the ACi group had a much higher incidence of PVB (23.5±5.3 counts/hour in week 1) and the frequency increased as HF developed (51.3±10.1 count/hour in week 4). CGP treatment significantly reduced the baseline frequency of PVB as compared to ACi (ACi+CGP PVB frequency: 9.2±6.3 counts/hr in week 1 and 13.0±3.7 counts/hr in week 4). Isoproterenol increased PVB frequency in all groups. At week 1, the PVB frequency in SHAMi after isoproterenol (1 mg/kg) was 104.0±36.2 counts/hour, while in ACi, the PVB frequency increased to 388.4±35.1 counts/hour after isoproterenol injection (Figs. 3A and 3B, right panels). CGP treatment inhibited the arrhythmogenic effect of the β-adrenergic challenge: PVB frequency following isoproterenol injection in the ACi+CGP group was 219.8±31.2 count/hour, a decrease of 43% compared to the ACi group. Although, as mentioned above, the HR response to isoproterenol was similar in all of the groups, the increase in PVB frequency following the injection was much less in week 4 than in week 1 (30.6±8.1 counts/hr in SHAMi, 105.9±12.5 in ACi, and 32.5±8.0 in ACi+CGP)(Fig. 3A and 3B, right panels), even though the isoproterenol dose was higher (2mg/kg). Increased intrinsic sympathetic drive related to post-operative stress in week 1 or β-adrenergic receptor desensitization in week 4 might explain this difference.

The antiarrhythmic effect of CGP was greater in week 4, both at baseline and after isoproterenol injection (Fig. 3A and 3B). CGP treatment (comparing ACi+CGP with ACi) suppressed baseline PVB frequency by 60% in week 1 and 75% in week 4. In the context of acute isoproterenol injection, CGP decreased PVB frequency by 43% in week 1 and 67% in week 4. Analysis of ECG data revealed prolonged QT intervals (QTc is corrected for HR) in both ACi and ACi+CGP groups (Fig. 3C). There was no difference in QTc between ACi and ACi+CGP groups at either week 1 or week 4, indicating that CGP treatment did not reverse QT prolongation in the HF model (Fig. 3C; right panels). QTc was also unaffected by the daily β-adrenergic challenge; there was no difference in QTc interval between SHAM and SHAMi groups.
Prevention of HF-associated SCD by CGP-37157 and overall mortality benefit.

The ACi model was associated with a remarkably high incidence of SCD. 61.3% of ACi animals died by the end of 4 weeks (Fig. 4) without overt HF symptoms (e.g., cyanosis, labored breathing, inactivity, piloerection or cachexia). ECG analysis showed that death was preceded by sustained polymorphic ventricular tachycardia and/or fibrillation (VT/VF) (Fig. 3A and 3B; left panels). Interestingly, SCD did not occur during the peak of the HR response after isoproterenol injection, but usually happened several hours after the injection (5/6 SCD events occurred between 2-5 hrs post injection, 1 occurred 9 hrs post-injection), when the HR had recovered almost to baseline. CGP treatment (ACi+CGP) significantly reduced the 4-week death rate to just 14.3%, which was not significantly different from that of the SHAMi control (Fig. 4). Moreover, all of the deaths in CGP-treated group and in the SHAMi group occurred in the first week after surgery, perhaps attributable to the post-operative stress making the animals more susceptible to arrhythmias. Survival analysis of the ACi group indicated that the death rate was higher in week 1 (22.4%) and week 4 (26.9%) compared to that in week 2 (15.8%) and week 3 (18.8%). Similar to the other groups, the higher death rate in week 1 in ACi may reflect the overall effects of post-operative stress, along with a higher susceptibility to isoproterenol-induced arrhythmias. Considering that the PVB frequency following isoproterenol injection in week 4 is only 27% of that in week 1, the higher death rate (26.9%) in week 4 suggests that PVBs may be more life-threatening due to HF-associated changes in the arrhythmogenic substrate.

Antiarrhythmic effects of CGP-37157 on the isolated failing heart.

We next determined if arrhythmia susceptibility and cardiac function in ACi failing hearts could be rescued by acute treatment with 1 μM CGP-37157 in isolated perfused hearts. Langendorff-perfused SHAMi and ACi hearts, isolated at week 4, were challenged with 10μmol/L isoproterenol for 15 min. Cardiac contractility and ECG were analyzed before (pre-ISO), during (ISO), and after (post-ISO) isoproterenol challenge. Baseline contractility before isoproterenol was significantly reduced in ACi hearts (Fig. 5A); LVDP, +dP/dt, and –dP/dt of ACi hearts were only 37%, 38%, and 36%, respectively, of values in SHAMi hearts (Table 1). Administration of isoproterenol increased cardiac contractility by a similar percentage in both ACi and SHAMi groups. However, ACi hearts were more sensitive to the stress of isoproterenol challenge; contractility of the ACi hearts was depressed more following isoproterenol washout. In the post-ISO state, LVDP, +dP/dt, and –dP/dt of ACi hearts were 12%, 16%, and 16% of values in SHAMi hearts (Table 1). Treatment with CGP-37157 had no statistically significant effects on cardiac contractility during pre-ISO or ISO states, but attenuated contractile dysfunction following isoproterenol treatment (Table 1). Treatment with CGP-37157 improved LVDP, +dP/dt, and –dP/dt recovery in the post-ISO state by 205%, 135%, and 120%, as compared to ACi hearts without CGP-37157 treatment (Table 1).

Heart rate variability (HRV) analysis was applied to quantify changes in the ECG in the SHAMi and ACi hearts under the different experimental conditions. Treatment with 10μmol/L isoproterenol did not induce overt arrhythmias during the period of maximum LVPD in either group. There were no differences in RR interval, standard deviation of RR interval (SDRR), root mean square of successive difference (rMSSD), short term dispersion (SD1), or long term dispersion (SD2) between SHAMi, ACi, and ACi+CGP treatment groups during pre-ISO and ISO states (Table 2). However, following washout of isoproterenol, HRV of ACi hearts was dramatically increased compared to that of SHAMi hearts (clusters of points are indicative of ectopic activity in the Poincaré plots in Fig. 5B), whereas CGP treatment completely inhibited the arrhythmias in ACi hearts in the post-ISO state (Fig. 5B and Table 2). There were no differences in RR interval, SDRR, rMSSD, SD1, or SD2 between SHAMi and ACi+CGP treated hearts (Table 2).
DISCUSSION

The major findings of the present study were that: 1) the transition from compensated pressure-overload hypertrophy to HF is exacerbated by a daily β-adrenergic challenge; 2) SCD is a major contributor to mortality in the ACi HF model; 3) the mitochondrial Na+/Ca2+ exchange inhibitor, CGP-37157, prevents cardiac contractile decompensation, blunts hypertrophic remodeling, decreases arrhythmia incidence, and prevents SCD associated with HF.

The present study was motivated by our earlier findings that cytoplasmic [Na+]c overload, by increasing the driving force for mNCE-mediated Ca2+ extrusion from the mitochondrial matrix, results in insufficient [Ca2+]m accumulation during increased work17,19,30. If the Ca2+ signal to the mitochondria falls below a critical threshold level19, Ca2+-dependent activation of Krebs cycle enzymes is disrupted29, causing the mitochondrial pyridine nucleotide redox potential (NAD(P)H/NAD(P)+) to become more oxidized. The consequent decrease in reducing equivalents driving oxidative phosphorylation (NADH) and the antioxidant pathways (NADPH) causes both an impairment of energy supply and ROS overload (Fig. 1 C and D). This pathological mechanism was demonstrated in normal myocytes overloaded with [Na+]c, myocytes treated with the Na+/K+ inhibitor ouabain30, and in myocytes isolated from failing (AC model) guinea pig hearts19. In the present study, we demonstrated that the net oxidation of NAD(P)H pool and increased oxidative stress during rapid pacing (4 Hz) in the presence of isoproterenol is also present in the ACi model. The increase in NAD(P)H oxidation, ROS accumulation, and arrhythmias associated with [Na+]c overload, can be prevented by either lowering [Na+]c or by inhibition of mNCE with CGP-3715719,30,31 (and the present work; Fig. 1C and D).

The findings are the first to demonstrate that chronic treatment with CGP-37157 prevents the progressive decline in contractile function and SCD in vivo in an animal model of HF. The ACi model was developed to incorporate both hemodynamic stress (left ventricular pressure overload) and chronic sympathetic hyperstimulation, two factors which are known to contribute to HF and SCD in humans32,33. Compared to pressure overload alone, the ACi model accelerated the decline in function and pathology of HF, while isoproterenol treatment in the absence of pressure overload (SHAMi) had no significant effects on cardiac function and remodeling in control animals.

A unique feature of the ACi model is the high incidence of SCD (~60% at 4 weeks), which is manifested as an increased death rate even in the first week post-aortic constriction, when function is largely preserved, and continues over the course of heart failure progression. Notably, the ACi model shows QTc prolongation even after only 1 week of pressure overload, prior to overt contractile dysfunction, as well as a large increase in spontaneous premature ventricular beats. The PVB rate and the incidence of VT/VF were both suppressed by CGP-37157 treatment; however, the QTc prolongation was not reversed, suggesting that the alterations in sarcolemmal ionic currents during hypertrophy, by themselves, are not sufficient to account for the increased automaticity and tendency towards reentry underlying SCD. Presumably, the combined effects of ion channel remodeling, oxidative stress, and the change in the myocardial substrate were all involved in setting the stage for VT/VF. In this light, it is still not clear if long QTc is a useful predictor of SCD in human HF. Breidthardt et al134 reported prolonged QTc interval (≥440ms) in 72% of patients with acute destabilized heart failure, but 720-day all-cause mortality was no different in patients with prolonged versus normal QTc intervals. Patients with prolonged QRS interval, however, had a significant 2-fold increase in mortality. Similarly, QTc was determined to have no independent prognostic value on 1 year mortality in a subset of patients with congestive heart failure in the DIAMOND trial135. In general, though, the overall risk of malignant arrhythmias increases with the magnitude of QTc prolongation36 and more refined analyses, such as QTc variability, may have predictive value for SCD37, perhaps as a better indicator of QT dispersion38.

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The present results suggest that prolonged QTc may provide the substrate for potentially fatal arrhythmias, but that a secondary event - we propose an oxidative stress event secondary to an energy/redox supply and demand mismatch - would be required to initiate SCD. The non-linear nature of the secondary event could therefore account for the unpredictability of SCD.

Apart from genetically engineered animals, the ACi model is, to our knowledge, one of the few animal models of HF-associated acquired long QT that displays a high incidence of spontaneous SCD. The accelerated transition between the compensated and decompensated states should be useful for investigating the mechanisms behind progressive systolic and diastolic dysfunction, as well as vulnerability to arrhythmias, in future studies.

Altered β-adrenergic receptor (β-AR) signaling is known to be a key player in the development of HF. In addition to impairment of the inotropic, chronotropic and lusitropic effects of β-AR stimulation in HF, chronic hyperactivation of the receptor results in receptor desensitization, detrimental effects on cardiac function, and structural remodeling, which can be mitigated by β-blockade. The impact of selective β-AR signal activation on the heart depends on the dose and duration of β-AR agonist administration. In our study, the chronic effects of isoproterenol on cardiac remodeling were minimized by selecting an isoproterenol dose that had no independent hypertrophic effects in sham operated animals and by administrating isoproterenol with repeated injections that induced only transient activation of β-AR signaling. The β-AR challenge had detrimental functional effects (arrhythmias and impaired post-ISO contractility) on LV pressure-overloaded animals, but not on controls, suggesting that the pressure-overloaded heart is more vulnerable to β-AR activation. This is consistent other animal studies. For example, in spontaneously hypertensive rats with LVH, infusion of isoproterenol initiated a transition from compensated hypertrophy to heart failure, whereas the same infusion had no significant effects on cardiac function and remodeling in control rats. Genetically-modified mouse lines that have constitutively increased β-AR drive with normal cardiac function and structure are also more susceptible to pressure overload. Our finding that CGP-37157 treatment protects pressure overloaded hearts (both in vivo and ex vivo) from the detrimental effects of β-AR challenge supports the hypothesis that blunted [Ca\textsuperscript{2+}]\textsubscript{m} accumulation leading to oxidative stress is a key event underlying increased vulnerability of the failing heart to β-AR-mediated cardiac dysfunction.

In addition to exacerbating contractile dysfunction, β-AR challenge also predisposed the pressure overloaded heart to VT/VF, and CGP-37157 protected ACi animals from both high PVB rate and SCD. Increased sympathetic activity during physical or emotional stress is known to provoke VT/VF in catecholaminergic polymorphic ventricular tachycardia (CPVT), and elevated diastolic Ca\textsuperscript{2+} and hyperactive ryanodine receptors (RyR) are thought to be critical factors leading to SCD in CPVT animal models. β-AR stimulation can contribute to arrhythmias by increasing Ca\textsuperscript{2+} current and increasing sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) activity, both of which increase SR Ca\textsuperscript{2+} load to increase vulnerability to spontaneous Ca\textsuperscript{2+} release. In HF, although the Ca\textsuperscript{2+} transient amplitude and SR load is decreased, the prolonged duration of the AP and Ca\textsuperscript{2+} transient, together with increased Ca\textsuperscript{2+} leak via the RyR, could favor spontaneous afterdepolarizations. For example, DeSantiago et al. demonstrated that increased SR Ca\textsuperscript{2+} by β2-AR activation results in enhanced arrhythmogenesis in myocytes from human and rabbit failing hearts. Interestingly, in the ACi model, the pro-arrhythmic effect of β-AR challenge was not evident during the peak of the response, but was revealed only after recovery from the increased work. We propose that CGP-37157 is protecting against damage induced by mitochondrial ROS accumulation by preserving the pyridine nucleotide/antioxidant capacity during β-AR activation. Among the potential ROS targets, the local oxidation of the RyR by mitochondrial ROS could underlie spontaneous Ca\textsuperscript{2+} release and ectopic activity. RyR channels have multiple cysteine residues that sense the local redox state, and their oxidation is responsible for increased spontaneous SR Ca\textsuperscript{2+} leak in HF, which can be normalized by dithiothreitol. In this light, we have previously demonstrated that CGP-37157, by increasing the....
mitochondrial Ca^{2+} response and preserving NAD(P)H redox potential during increased work, can prevent DADs triggered by ouabain-induced Na\(^+\) overload in myocytes, hearts, and intact animals\(^{30}\). This mechanism is the most likely explanation for the CGP-mediated protection against post-ISO ectopic activity observed in the isolated perfused ACi hearts (Fig. 5).

In addition to ROS-driven dysfunction, impaired mitochondrial energetics in HF also contributes to cardiac decompensation\(^{50}\). For example, limitations of ATP delivery could impair diastolic function by affecting the Ca\(^{2+}\) removal capacity via SERCA when energy demand increases during β-AR stimulation. This has been observed in studies of energetically-deficient animal models. In the creatine kinase deficient mouse, the reduced energy reserve leads to a decreased capacity of SERCA to remove cytosolic Ca\(^{2+}\) \(^{51,52}\). A peroxisome proliferator-activated receptor gamma coactivator 1-β (PGC-1β) knockout mouse also displays reduced respiration rate and ATP production\(^{53}\); these animals showed increased susceptibility to catecholamine-induced arrhythmias\(^{54}\). Cardiomyocytes from these mice have normal Ca\(^{2+}\) transients at rest, but elevated [Ca\(^{2+}\)]c and spontaneous diastolic Ca\(^{2+}\) transients were induced following isoproterenol administration\(^{54}\). By improving energy supply, CGP-37157 treatment could thus help to maintain the activity of SERCA or other energy-dependent processes during increased work to preserve systolic and diastolic function.

The present findings highlight the central role of oxidative stress in both the functional deterioration and arrhythmias associated with HF progression. In addition to direct damage to the myocyte, mounting evidence indicates that ROS modulate various signaling pathways involved in hypertrophic growth, apoptotic and necrotic cell death, and proliferation of cardiac fibroblasts\(^{11,55,56}\). ROS are also downstream mediators of neurohumoral stimuli implicated in HF, such as angiotensin II, endothelin 1, and tumor necrosis factor α. Increased ROS can also directly activate signaling cascades including tyrosine kinases, MAP kinases, PKC, and PI3-kinase\(^{57}\), which are potent regulators of cardiac growth. The effects of CGP on these downstream effectors of ROS signaling remain to be determined, but could also contribute to the observed protection. Nevertheless, given that the hypertrophic response was only partially decreased by CGP treatment, the major beneficial effect of CGP may be to prevent the redox modification of proteins, (e.g. ion channels, myofilament proteins, and mitochondrial enzymes) that lead to contractile dysfunction and deficient energetics. Recent studies confirm the preeminent role of mitochondrial ROS in HF, for example, a mitochondrially-targeted antioxidant peptide (SS-31) protects animals from Ang II-induced cardiac hypertrophy and fibrosis\(^{14}\) and overexpression of mitochondrial, but not cytosolic, catalase prevents changes in the proteome of mice subjected to pressure-overload-induced heart failure\(^{15}\). CGP treatment intervenes in the mechanism leading to the mitochondrial ROS overload, while preserving energy supply and demand balance, which may have advantages over strategies that simply attempt to increase the ROS scavenger capacity.

In summary, CGP-37157 preserves cardiac function, attenuates remodeling and fibrosis, and prevents SCD in the guinea pig heart failure and SCD model produced by pressure overload combined with β-AR stimulation. The beneficial effects of CGP-37157 can attributed to the effects of restored [Ca\(^{2+}\)]m dynamics by CGP-37157 on mitochondrial energetics and redox balance. Additional studies will be needed to characterize all of the HF-associated modifications impacted by CGP-37157 treatment, including cellular redox status, ROS balance, the electrophysiology of the heart, excitation-contraction coupling, energy metabolism, and post-translational protein modifications. More work will also be required to determine if CGP treatment can reverse established left ventricular hypertrophic remodeling to prevent decompensation and SCD in this model. In addition, given the known variations in cytoplasmic Na\(^+\) in different species\(^{58}\), the efficacy of CGP may be different in other models of HF and in humans. Nevertheless, the findings support the hypothesis that [Ca\(^{2+}\)]m dynamics plays a critical role in the development of HF and HF-associated SCD and implicate mNCE as a novel therapeutic target for HF.
SOURCES OF FUNDING
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DISCLOSURES
None

REFERENCES
41. Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart
42. Osadchii OE. Cardiac hypertrophy induced by sustained beta-adrenoreceptor activation: pathophysiological aspects. Heart Fail Rev. 2007;12:66-86
47. Wehrens XH, Marks AR. Altered function and regulation of cardiac ryanodine receptors in cardiac disease. Trends Biochem Sci. 2003;28:671-678
57. Sabri A, Hughie HH, Luechesi PA. Regulation of hypertrophic and apoptotic signaling pathways by reactive oxygen species in cardiac myocytes. Antioxid Redox Signal. 2003;5:731-740
FIGURE LEGENDS

Figure 1. The effects of CGP-37157 on [Ca²⁺]_m dynamics, NAD(P)H oxidation, and ROS production in myocytes isolated from ACi hearts. A) Representative recording of MityCam fluorescence (1-F/F₀) in cardiomyocytes isolated from SHAMi (black) and ACi with (light gray) or without (gray) the presence of 1µmol/L CGP-37157 (CGP) in the perfusate. B) Mean peak MityCam fluorescence (1-F/F₀) during 1Hz stimulation in SHAMi (n=30), ACi (n=29), and ACi + CGP (n=13). *, p<0.001 as compared to SHAMi; **, p<0.001 as compared to ACi. In the presence of 100nmol/L isoproterenol, NAD(P)H autofluorescence (A) and CM-DCF oxidation (B) were recorded in myocytes isolated from SHAMi hearts (n=3) or ACi hearts, with (n=3) or without (n=3) 1µmol/L CGP-37157 (CGP) in the perfusate. Box indicates duration of 4Hz field-stimulation.

Figure 2. The effects of CGP-37157 treatment on hypertrophic remodeling and cardiac function. A) Heart weight, HW (left panel), and Lung weight, LW (right panel), were measured at 4 weeks after surgery and normalized to tibia length (TL). Isoproterenol challenge had no effects on either HW/TL or LW/TL in sham-operated animals (SHAMi vs. SHAM). Pressure overload with daily isoproterenol challenge (ACi) induced cardiac hypertrophy and lung congestion, and CGP-37157 treatment attenuated both cardiac hypertrophy and lung congestion (ACi+CGP). B) Interstitial fibrosis was analyzed with Masson’s Trichrome staining. Left panel: representative images from each experimental group; right panel: measurements of interstitial fibrous tissue showing that pressure overload, together with isoproterenol challenge, induced significant fibrosis in ACi group, which was attenuated by CGP-37157 treatment (ACi+CGP). C) Cardiac function is preserved by CGP-37157. Upper panel: representative pictures of echocardiographic study; lower panel: measurements of fractional shortening (FS) (left) and diastolic LV internal dimension (LVIDd) (right) showing reduced FS and LV dilation in ACi group but not in CGP-37157 treated group. D) Left panel: representative LV pressure-volume loops. Heart from ACi group shows increased end-diastolic volume and depressed end-systolic pressure-volume relation. Right panel: summary data of end-systolic LV pressure (upper panel) and dP/dt_ip (dP/dt normalized to pressure) (lower panel). Isoproterenol treatment in the absence of aortic constriction (SHAMi) had no effect on cardiac function.

Figure 3. The antiarrhythmic effect of CGP-37157. A and B) Left panels: Representative telemetric ECG recordings at week 1 (A) and week 4 (B) after aortic constriction showing baseline records before isoproterenol injection, records after isoproterenol injection (post-ISO), and during recovery 4 to 5 hours after isoproterenol injection. Black arrows indicate PVBs; white arrows indicate VF. Right panels: PVB incidence at baseline and after isoproterenol injection in week 1 and week 4. *, p<0.05 compared to SHAMi group, †, p<0.05 ACi+CGP compared to ACi group. C) Left panels: representative ECG recordings at baseline for each group at weeks 1 and 4; right panels: summary measurements of QTc at week 1 and week 4 showing that long QT was induced by pressure overload. Neither CGP-37157 treatment nor isoproterenol challenge had any effect on QTc.

Figure 4. Survival curves over 4 weeks after the time of surgery for animals in SHAM, SHAMi, ACi, and ACi+CGP groups. Survival of animals with pressure overload plus isoproterenol challenge was significantly increased by treatment with CGP-37157.

Figure 5. Effects of acute CGP-37157 treatment on isolated perfused failing hearts. A) Representative traces of LV pressure and ECG simultaneously recorded before (pre-ISO), during (ISO), and after (post-ISO) isoproterenol (10µM) exposure in hearts of shami (n=3), ACi (n=3), and ACi with CGP-37157 (1 µM) treatment (ACi+CGP) (n=3). B) Representative Poincaré plots of SHAMi, ACi, and ACi+CGP hearts during pre-ISO, ISO, and post-ISO states, respectively. Ectopic activity is evident as clustered of points only in the ACi heart post-ISO (middle right panel).
Table 1. Measurements of cardiac contractility in isolated perfused heart.

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<th>Shami</th>
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<th>ACi+CGP</th>
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<tr>
<td></td>
<td>Pre-iso</td>
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<td>Post-iso</td>
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<td>+dP/dt</td>
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*, p<0.05 when compared to contrl; †, p<0.05 when compared to ACi.

Table 2. HRV analysis. (SDNN, standard deviation of normal RR interval; RMSSD, root mean square of the successive differences)

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<tr>
<td></td>
<td>Time domain</td>
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<td>Mean RR (ms):</td>
<td>260±18</td>
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<td>SDNN (ms):</td>
<td>3.85±1.39</td>
<td>42.08±8.51*</td>
<td>2.76±1.69†</td>
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<td>RMSSD (ms):</td>
<td>0.88±0.29</td>
<td>65.60±15.60*</td>
<td>1.14±0.59†</td>
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<td>Nonlinear Results</td>
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<td>SD1 (ms):</td>
<td>0.626±0.207</td>
<td>46.418±11.048*</td>
<td>0.804±0.415†</td>
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<td>SD2 (ms):</td>
<td>5.394±1.968</td>
<td>36.542±6.167*</td>
<td>3.812±2.347†</td>
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</table>

*, p<0.05 when compared to contrl; †, p<0.05 when compared to ACi.
Novelty and Significance

What Is Known?

- Sudden cardiac death (SCD) accounts for up to 50% of deaths in patients with heart failure (HF) and, in many cases, can be attributed to paroxysmal ventricular arrhythmias.
- The mechanisms underlying SCD are unknown but ion channel remodeling, action potential prolongation, and abnormal cytoplasmic and mitochondrial Ca\(^{2+}\) handling have been implicated.
- Energy metabolism is also known to be altered in HF but the links between impaired energy supply, oxidative stress, and contractile and electrical dysfunction are incompletely understood.

What New Information Does This Article Contribute?

- A novel guinea-pig model of HF/SCD is described, combining pressure-overload and daily catecholamine challenge, which displays impaired contractility, long-QT, and a high incidence of SCD due to spontaneous ventricular fibrillation over a 4-8 week time span.
- Treatment with an inhibitor of the mitochondrial Na\(^+/\)Ca\(^{2+}\) exchanger (mNCE) prevents contractile decompensation and SCD in vivo but does not prevent QT prolongation.
- mNCE inhibition prevents NAD(P)H oxidation and ROS overload during increased work in myocytes from failing hearts and decreases the incidence of arrhythmias after a catecholamine challenge in perfused failing hearts.
- Impaired mitochondrial Ca\(^{2+}\) balance, and the consequent impairment of NAD(P)H availability, plays a key role in heart failure progression and vulnerability to fatal arrhythmias by compromising energy supply and antioxidant capacity in the failing heart.

Sudden cardiac death is a major cause of death in patients with heart failure, but the mechanisms underlying this increased vulnerability are poorly understood. In cardiomyocytes from failing hearts, it was previously shown that insufficient mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{mit}}\)) accumulation, secondary to cytoplasmic Na\(^{+}\) overload, decreases NAD(P)H/NAD(P)\(^+\) redox potential, increases oxidative stress, and disrupts Ca\(^{2+}\) handling when workload increases. Here, we show that chronic in vivo treatment with an inhibitor of the mitochondrial Na\(^+/\)Ca\(^{2+}\) exchanger (CGP-37157) prevents the progression from compensated hypertrophy to heart failure and eliminates the high incidence of spontaneous SCD. The findings highlight the critical role played by mitochondrial Ca\(^{2+}\) dynamics in maintaining redox and energy balance in the failing heart and support the feasibility of targeting mitochondria for therapeutic intervention in heart failure.
Figure 1

A. MityCam signal (1-F/F0) over time (sec).

B. Maximum 1-F/F0 for SHAMi, ACi, and ACi+CGP.

C. Reduced NADH (%) in different conditions.

D. DCF (F/F0) over time (sec) for SHAMi, ACi, and ACi+CGP.
Figure 2
Figure 3

**Week 1**

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**Week 4**

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**SHAMi**

**ACi**

**ACi+CGP**

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**C**

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**Week 1**

**Week 4**

---

**Baseline PVB (counts/hour)**

- **SHAMi**: 0, 30, 60, 90, 120
- **ACi**: 0, 30, 60, 90, 120
- **ACi+CGP**: 0, 30, 60, 90, 120

**Post-ISO PVB (counts/hour)**

- **SHAMi**: 100, 200, 300, 400
- **ACi**: 100, 200, 300, 400
- **ACi+CGP**: 100, 200, 300, 400

---

**Baseline QTc (ms) at week 1**

- **SHAM**: 0, 50, 100, 150, 200
- **SHAMi**: 0, 50, 100, 150, 200
- **ACi**: 0, 50, 100, 150, 200
- **ACi+CGP**: 0, 50, 100, 150, 200

**Baseline QTc (ms) at week 4**

- **SHAM**: 200, 250, 300, 350
- **SHAMi**: 200, 250, 300, 350
- **ACi**: 200, 250, 300, 350
- **ACi+CGP**: 200, 250, 300, 350

---

*Significant difference
†Trend towards significance
Figure 4

![Survival Analysis Graph](image)

- **SHAM** ($n=12$)
- **SHAMi** ($n=20$)
- **ACi** ($n=49$)
- **ACi+CGP** ($n=28$)

$\text{Survival (\%)}$

$\text{Day}$

$p<0.01$
Figure 5

A

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ACi

| mmHg     | ✩     | ✩       | ✩  | ✩        |
| mV       | ✩     | ✩       | ✩  | ✩        |

ACi+CGP

| mmHg     | ✩     | ✩       | ✩  | ✩        |
| mV       | ✩     | ✩       | ✩  | ✩        |

B

SHAMi

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ACi

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ACi+CGP

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Inhibiting Mitochondrial Na⁺/Ca²⁺ Exchange Prevents Sudden Death in a Guinea Pig Model of Heart Failure

Ting Liu, Eiki Takimoto, Veronica L Dimaano, Deeptankar DeMazumder, Sarah Kettlewell, Godfrey L Smith, Agnieszka Sidor, Theodore Abraham and Brian O'Rourke

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Supplemental Materials

Methods

*Animal model:* Hartley guinea pigs (~300 g; HillTop Lab Animals) were housed in an animal facility at the Johns Hopkins University. This study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Johns Hopkins Animal Care and Use Committee.

Male animals were anesthetized with 4% isoflurane in a closed box for 4 min, and then intubated. Animals were ventilated with oxygen and 2% isoflurane. Ascending aortic constriction (AC) was produced by tying a suture around the ascending aorta using an 18-gauge needle as a spacer, which was then removed. Sham-operation was performed following the same procedure without tying the suture. When animals were breathing spontaneously after the procedure, bupronex (0.05mg/kg) was administrated via intramuscular injection for analgesia and animals were observed until full recovery. Isoproterenol was administrated daily by i.p. injection at 1 mg/kg for the first week after surgery and at 2 mg/kg for another 3 weeks. In the group with CGP treatment, CGP was introduced by i.p. injection (~40mg/kg) followed by implantation of an osmotic pump in the abdominal cavity (delivery rate: 0.015 mg/kg/hr) for extended delivery of the compound.

*Echocardiography:* Transthoracic echocardiography was performed in guinea pigs at 4 weeks after surgery. Following sedation with isoflurane inhalation (1 L/min, 1% to effect), the anterior chest area was shaved and the guinea pig was placed on a thermal pad in supine position with its limbs restrained. Electrocardiogram leads were fastened to the two front limbs and the left rear limb. Temperature was monitored via a rectal probe maintained at 37°C using a warming pad and a heating lamp. Heart rate was monitored, and echocardiography was performed using a 5-12 MHz ultraband transducer interfaced with Vivid 7 System only when heart rate was above 220 BPM. Two-dimensionally directed M-mode images were obtained from the long axis views. Echocardiographic measurements were made on 3 consecutive cardiac cycles by the leading edge-to-leading edge method. LV end-diastolic and end-
systolic dimensions, and LV end-diastolic posterior wall thickness, were measured from the M-mode images, and left ventricular fractional shortening was calculated with the software (VisualSonics V1.3.8).

Cardiac myocyte isolation: Cardiac myocytes were isolated from SHAMI and ACi guinea pig hearts by enzymatic digestion as described previously.1

Fluorescence recordings of mitochondrial Ca2+ dynamics, NADH, and ROS in isolated myocytes: Myocytes were loaded into a heated field-stimulation chamber (37°C) on the stage of a fluorescence microscope (Nikon Eclipse TE300) and superfused with Tyrode’s solution containing (in mmol/L): NaCl 130, KCl 5, MgCl2 1, Na-HEPES 10, CaCl2 2, glucose 10, pyruvate 2, ascorbic acid 0.3; pH 7.4. [Ca2+]m was measured with a genetically-encoded, mitochondrially-targeted Ca2+ indicator, MityCam2. Adenovirus-mediated MityCam gene transduction was performed in vivo by intramuscular injection of 100µl adenovirus suspension (1-5X1010 virus particles) into the LV free wall of ACi or SHAMI guinea pigs at week 3 after aortic constriction surgery. Cardiomyocytes were then isolated at 5-7 days later. In the presence of 100nM isoproterenol, cells with MityCam expression were imaged every 25ms at resting state followed by 0.1 and 1Hz stimulation, respectively, and then returned to resting state. MityCam fluorescence was measured with Image J (NIH) and expressed as 1-F/F0 (F0 was the baseline fluorescence prior to stimulation). The level of [Ca2+]m accumulation was taken as the peak 1-F/F0 of MityCam fluorescence during 1hz stimulation. The endogenous autofluorescence of NAD(P)H was recorded with 360nm excitation and emission at 450nm. NAD(P)H redox potential was expressed as percent reduction of the NAD(P)H/NAD(P)+ pool, calibrated by applying the mitochondrial uncoupler FCCP (5 µM; 0% reduced) and the cytochrome oxidase inhibitor NaCN (4 mM; 100% reduced) at the end of each recording. To monitor ROS production, myocytes were loaded with 2µM CM-DCFDA, CM-DCF fluorescence was excited 485nm, and its emission was measured at 525nm. In the presence of 100nM isoproterenol, fluorescence was recorded for 100s in the resting state and 100s at 4 Hz stimulation followed by another 100s recording in the resting state. For CGP-37157 treatment, cells were perfused with 1 µM CGP-37157 for 5 min before recording.

In vivo hemodynamic study: Guinea pigs were anesthetized and ventilated as described above. The external jugular vein was cannulated with 30-gauge needle for volume administration. Fluid
supplementation (25% human albumin in saline) was provided at 100 µl/min. The apex portion of the heart was exposed via a substernal-transverse incision. The pericardium was opened at the apex, and an apical stab will be made with a 25-gauge needle to place a 1.4-F, four-electrode pressure-volume catheter (model SPR-719, Millar Instruments) along the long axis. The pressure-volume catheter was connected to a custom-designed conductance system producing a constant current of 30 µA at a frequency of 2 or 20 kHz. Correct catheter positioning was confirmed by on-line visualization of the pressure-volume loops and placement of the distal electrode within the chamber. After a short stabilization period, left ventricular (LV) pressure–volume loops were recorded at baseline for 5 s with the ventilator stopped (typically 20-30 cardiac cycles), and then the pressure-volume relationship was measured by transiently occluding the inferior vena cava. At the end of each experiment, a bolus of hypertonic saline was rapidly injected into jugular vein to determine the parallel conductance volume coefficient (Vp) and absolute volume, and aortic flow was measured by insertion of an ultrasonic perivascular flow probe connected to a flow meter (probe: model 1RB, flow meter: T106, Transonics, Ithaca, NY) around the abdominal aorta, which was used to determine stroke volume. Data were collected and analyzed using custom-developed software.

**Histological study:** Hearts were excised and rapidly immersed into ice-cold saline solution. The aorta was then cannulated with a 16G needle, retrogradely perfused with 4% phosphate-buffered paraformaldehyde, and immersion-fixed overnight in the same fixative. Following fixation, the specimens was embedded in paraffin and 5 µm sections were cut perpendicular to the long axis of the heart. 6 sections were collected from the mid-ventricular region of each heart, and stained with Masson’s Trichrome to assess the interstitial fibrosis. Interstitial collagen fraction was determined using computer-assisted image analysis (NIH Image J).

**Electrocardiogram analysis:** ECG data were collected with a telemetry system (DSI, St. Paul MN). An ECG transmitter (EA-F20) was implanted in the abdominal cavity of the animal and the leads were secured in a lead II placement at the end of AC surgery. ECG signals were collected and transmitted via radio-frequency signals to a receiver that was connected to a computer, and the data was exported and analyzed with the Dataquest OpenART software system.
Hemodynamics and ECG study in Langendorff-perfused hearts: The ex vivo study was performed 4 weeks after surgery. Recordings of hemodynamics and ECGs were made in Langendorff-perfused ACi or SHAMi hearts as previously described. After a 10min equilibration period, hearts were subjected to the following protocol: 10 minute baseline recording while the heart was perfused with control buffer, application of 10 μM isoproterenol and 15 minutes recording, washout with control buffer with recording for another 20 minutes. In the CGP treated group, 1 μM CGP was added to the buffer at the beginning of baseline recording. The measurements of hemodynamic parameters were determined by taking the average during the last 5 minutes before isoproterenol application (pre-ISO), for 3 minutes during the maximal effect on LV developed pressure (LVDP) of isoproterenol (ISO), and for 5 minutes at 15 minutes after isoproterenol washout (post-ISO). Simultaneously recorded ECG data during the same time periods were used for blinded HRV analysis.

Statistical Analysis: Data are expressed as mean ± SEM. Comparisons between 2 groups were performed with unpaired t-test. Survival rates were analyzed with Kaplan-Meier method. Sample sizes are provided in the Figures.

Methods used for supplemental experiments:

Effects of CGP-37157 on NCX current (I_{NCX}) and action potential (AP) – Myocytes were loaded in a heated chamber (37°C) on the stage of a fluorescence microscope (Nikon Eclipse TE300) and were whole-cell patch-clamped with 2-4 MΩ pipettes. For I_{NCX} measurement, cells were superfused with Cs-Tyrode’s solution, containing (in mM): NaCl 130, CsCl 5, MgCl₂ 1, Na-HEPES 10, CaCl₂ 2, glucose 10; pH 7.4. To block L-type Ca²⁺ channels and Na⁺-K⁺-ATPase, 10μM nitrendipine and strophanthidin, respectively, were applied in Cs-Tyrode’s solution. Cells were voltage-clamped and equilibrated with a pipette solution containing (in mM) CsCl 120, MgCl₂ 0.5, Na-HEPES 20, Mg-ATP 5, BAPTA 5, and CaCl₂ 3; pH7.25. The presence of 5mM BAPTA and 3mM CaCl₂ in pipette solution buffered the intracellular Ca²⁺ to 200nM. After achievement of whole-cell conformation, cells were voltage-clamped at a holding potential of -
40mV. $I_{\text{NCX}}$ was recorded with families of pulses applied from +80 to -80 mV in 20 mV steps for 300 ms at 0.5 Hz. $I_{\text{NCX}}$ was defined as the current blocked by 5 mM NiCl$_2$ (Ni-sensitive current). To record AP, cells were superfused with normal Tyrode’s solution and were current-clamped with a pipette solution containing (in mM): K-glutamate 120, KCl 10, HEPES 10, EGTA 5, MgATP 5, and pH 7.2. Cells were paced at 0.5Hz. 1 and 5µM CGP was applied after steady-state AP was achieved. AP duration was measured with custom software.

*Measurement of intracellular Na$^+$*: Intracellular Na$^+$ was measured ratiometrically using SBFI as described previously.  

**References:**


**ONLINE FIGURES:**
Online Figure I. Interactions between Ca\textsuperscript{2+} handling (green), mitochondrial oxidative phosphorylation (OxPhos; orange), and the antioxidant system (AOS; blue). During excitation-contraction coupling, L-type Ca\textsuperscript{2+} channels (L) trigger SR Ca\textsuperscript{2+} release channels (RyR) and activation of myofilament myosin ATPase activity. Increases in work (ATP demand) are associated with increases in mitochondrial Ca\textsuperscript{2+} uptake via the calcium uniporter MCU, leading to activation of NADH production by the tricarboxylic acid (TCA) cycle to match a higher NADH oxidation rate by the electron transport chain (complexes I, II, III, IV, V). NADPH in the mitochondrial matrix, derived from reactions dependent on TCA cycle intermediates and a transhydrogenase (THD), donates electrons to support the moiety-conserved glutathione, thioredoxin, and glutaredoxin antioxidant pathways. Increased cytoplasmic Na\textsuperscript{+} and blunted Ca\textsuperscript{2+} release in HF results in enhanced efflux through the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (mNCE), the target of CGP-37157, resulting in insufficient NAD(P)H production to maintain both ATP production and reactive oxygen species (ROS) scavenging. Mitochondrial ROS emission increases to affect multiple redox-sensitive targets in the cell, leading to arrhythmias, Ca\textsuperscript{2+} and contraction dysfunction, and cell death. NCX: sarcolemmal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, SERCA: SR/ER Ca\textsuperscript{2+} ATPase, GSH: Reduced glutathione, GSSG: oxidized glutathione GR: glutathione reductase, GPX: glutathione peroxidase, TrxSS: oxidized thioredoxin, Trx-SH: reduced thioredoxin, TrxR: thioredoxin reductase, Prx: peroxiredoxin, SOD: superoxide dismutase.
**Online Figure II.** A daily β-adrenergic challenge exacerbates functional deterioration in a pressure-overload model of heart failure. Cardiac fractional shortening (FS) measured with echocardiography at week 0, 2, 4, 6, and 8 after ascending aortic-constriction in guinea pigs with (ACi) or without (AC) daily isoproterenol injection. In ACi group, 4 of 6 animals died following overt HF symptoms before echocardiographic imaging at week 8 and the FS of these animals was counted as 0%. *, p<0.01 as compared to week 0 of the same group.
Online Figure III

A) Representative SBFI ratio images (340/380 nm exc.) of a cardiomyocyte when cell was intact (baseline) and when $[\text{Na}^+]_c$ was clamped to 0, 70, and 140 mM in the presence of 7.5 µM gramicidin D.

B) Average $[\text{Na}^+]_c$ measured in myocytes isolated from SHAMi (n=4) and ACi (n=17) hearts, * $p<0.001$ as compared to SHAMi.
Online Figure IV. Effect of CGP on $I_{\text{NCX}}$, APD, and QTc. A) To determine if CGP-37157 inhibits sarcolemmal NCX, $I_{\text{NCX}}$ was recorded with perfusate containing 0, 1, 5, or 10 µM CGP. Representative recording of sarcolemmal NCX activity. CGP of various concentrations and NiCl$_2$ (Ni) were added to perfusate as indicated. B) Measurements of Ni-sensitive $I_{\text{NCX}}$ at +80mV in the presence of 0, 1, 5, and 10 µM CGP (n=4). C) Time course of APD$_{50}$ and APD$_{90}$ shows that application of CGP 1 and 5µM has no effect on APD (n=3). D) The acute effect of CGP-37157 on QT intervals was examined in Langendorff-perfused hearts. There was no difference in the average QTc of a 3-min period before and after 1µM CGP administration (n=3).
Online Figure V. The chronotropic effect of β-adrenergic challenge. Heart rates (HR) of animals in SHAM (n=3), SHAMI (n=4), ACi (n=6), and ACi+CGP (n=4) groups were analyzed at week 1 (A) and week 4 (B) post-banding. Upper panels are representative HR recordings (normalized to baseline) for 12 hours showing the baseline before isoproterenol (iso) injection, the increase of HR in response to iso, and recovery from iso effect. Arrow indicates iso injection. Lower panels show average baseline HR and maximum HR following iso injection. *, p<0.05 compared to SHAMI group; †, p<0.05 ACi+CGP compared to ACi group.