Interventricular heterogeneity as a substrate for arrhythmogenesis of decoupled mitochondria during ischemia in the whole heart

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Smith RM, Velamakanni SS, Tolkacheva EG. Interventricular heterogeneity as a substrate for arrhythmogenesis of decoupled mitochondria during ischemia in the whole heart. Am J Physiol Heart Circ Physiol 303: H224–H233, 2012. First published May 25, 2012; doi:10.1152/ajpheart.00017.2012.—Myocardial ischemia results in metabolic changes, which collapse the mitochondrial network, that increase the vulnerability of the heart to ventricular fibrillation (VF). It has been demonstrated at the single cell level that uncoupling the mitochondria using carbonyl cyanide p-(tri-fluoromethoxy)phenyl-hydrazone (FCCP) at low concentrations can be cardioprotective. The aim of our study was to investigate the effect of FCCP on arrhythmogenesis during ischemia in the whole rabbit heart. We performed optical mapping of isolated rabbit hearts (n = 33) during control and 20 min of global ischemia and reperfusion, both with and without pretreatment with the mitochondrial uncoupler FCCP at 100, 50, or 30 nM. No hearts went into VF during ischemia under the control condition, with or without the electromechanical uncoupler blebbistatin. We found that pretreatment with 100 (n = 4) and 50 (n = 6) nM FCCP, with or without blebbistatin, leads to VF during ischemia in all hearts, whereas pretreatment with 30 nM of FCCP led to three out of eight hearts going into VF during ischemia. We demonstrated that 30 nM of FCCP significantly increased interventricular (but not intraventricular) action potential duration and conduction velocity heterogeneity in the heart during ischemia, thus providing the substrate for VF. We showed that wavebreaks during VF occurred between the right and left ventricular junction. We also demonstrated that no VF occurred in the heart pretreated with 10 μM glibenclamide, which is known to abolish interventricular heterogeneity. Our results indicate that low concentrations of FCCP, although cardioprotective at the single cell level, are arrhythmogenic at the whole heart level. This is due to the fact that FCCP induces different electrophysiological changes to the right and left ventricle, thus increasing interventricular heterogeneity and providing the substrate for VF.

myocardial ischemia; optical mapping; carbonyl cyanide p-(tri-fluoromethoxy)phenyl-hydrazone; mitochondria; interventricular heterogeneity

MYOCARDIAL ISCHEMIA IS CHARACTERIZED by a deficient energetic input as well as deficient waste removal (10). This results in a failure of contraction, deterioration of electrical behavior, and, if prolonged (>30 min), the eventual death of the cell (10), which can lead to lethal ventricular fibrillation (VF) or mechanical pump failure.

Under normal aerobic conditions, NADH and FADH₂, which are formed during glycolysis and the tricarboxylic acid cycle, transfer their electrons to O₂ through the electron transport chain (2, 26). This transfer sets up the chemiosmotic gradient that drives ATP synthesis through the F₁F₀-ATP synthase at the inner membrane of the mitochondria and therefore allows the mitochondria to maintain its membrane potential. During the early phase of ischemia, electron transport and the ejection of H⁺ in the mitochondria is ceased (4). As a consequence, the electrochemical gradient necessary for ATP synthesis is insufficient to keep up with the energy demands of the cell, resulting in depolarization of the mitochondrial membrane, which causes a significant drop in ATP and an increase in the intracellular Ca²⁺ concentration (4).

Previous studies have shown that if the heart is exposed to repeated brief periods of ischemia-reperfusion there is cardioprotection against prolonged ischemic injury (12, 21). It has been hypothesized that the mitochondria plays an important role in this cardioprotection mechanism (1, 43). Specifically, studies in single ventricular myocytes have shown that depolarizing the mitochondria using low concentrations (≤100 nM) of carbonyl cyanide p-(tri-fluoromethoxy)phenyl-hydrazone (FCCP), which dissociates the electron transport chain from ATP synthase (3, 49), leads to cardioprotection when administered before ischemic injury (8, 9). In single myocyte studies, cardioprotection was determined by examining mitochondrial oxidation and mitochondrial membrane potential (8); moreover, in their accompanying study in the whole rat heart, cardioprotection was determined by left ventricular end diastolic pressure (9). However, the cardioprotective effect of FCCP on the electrophysiological properties of the whole heart has never been demonstrated.

In this study, we aim to determine whether FCCP at low concentrations, shown to be cardioprotective in the single myocyte, is cardioprotective or arrhythmogenic in the isolated Langendorff-perfused rabbit heart during ischemia. In addition, we investigated the changes in various electrophysiological properties of the heart, during periodic pacing and VF, induced by uncoupling the mitochondria.

MATERIALS AND METHODS

All experiments were approved and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1996) and the University of Minnesota guidelines regulating the care and use of animals. New Zealand White rabbits (Bakkom Rabbitry, 1.3–2.0 kg, n = 33) were first heparinized (550 U/100 g) and then anesthetized with ketamine and xylazine (35 and 5 mg/kg, respectively). The heart was excised and placed in an ice-cold cardioplegia solution (5). Two green lasers (532 nm, 1 W; Shanghai Dream Laser) were used for excitation, and the fluorescence signal was recorded simultaneously.
from the right (RV) and left (LV) ventricular epicardial surfaces by two fast 12-bit charge-coupled device cameras (Dalsa, Waterloo, Ontario, Canada). A typical field of view of our two-camera system is shown in Fig. 2A. A dynamic pacing protocol was used to periodically stimulate the base of the heart at progressively reduced basic cycle lengths (BCLs) from 300 ms in steps of 20 ms until 200 ms, and then in steps of 10 ms until 160 ms. Optical movies of 1.6 s were acquired at 600 frames/s with 64 × 64 pixel resolution. The background fluorescence was subtracted from each frame, and spatial (3 × 3 pixels) and temporal (3 pixels) conical convolution filters were used.

Four protocols were used (Fig. 1): 1) control, no-flow global ischemia, and reperfusion (RP) (n = 10); 2) control, pretreatment with either 100, 50 (n = 10), or 30 (n = 8) nM of FCCP (8, 9), ischemia, and RP; 3) control, pretreatment with glibenclamide (Gmide; 10 μM) (15, 24, 34), ischemia, and RP (n = 3); and 4) control, Gmide (10 μM), FCCP (30 nM), ischemia, and RP (n = 3).

During no-flow ischemia, the bathing chamber was gassed with 95% N₂-5% CO₂ to minimize O₂ reaching the muscle surface. Drugs were delivered via perfusate, similar to the single cell studies (8). Optical movies were recorded at the following time points: 30 min of control, 5 min of FCCP pretreatment, 20 min of Gmide pretreatment, and 15 min of ischemia, and 30 min of RP. After our experiments, we performed 2,3,5-triphenyltetrazolium chloride (TTC) staining to show in Fig. 2A. A dynamic pacing protocol was used to periodically stimulate the base of the heart at progressively reduced basic cycle lengths (BCLs) from 300 ms in steps of 20 ms until 200 ms, and then in steps of 10 ms until 160 ms. Optical movies of 1.6 s were acquired at 600 frames/s with 64 × 64 pixel resolution. The background fluorescence was subtracted from each frame, and spatial (3 × 3 pixels) and temporal (3 pixels) conical convolution filters were used.

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To evaluate the effect of blebbistatin on the vulnerability of the heart to VF during ischemia, we performed experiments (n = 2) without blebbistatin. Additional experiments were performed with (n = 3) and without (n = 3) blebbistatin in the heart pretreated with 50 nM FCCP. In these experiments, the anterior surface of the heart was imaged allowing visualization of the RV-LV junction.

Parameter Measurements

Only those hearts that did not go into sustained VF during experiments were used for the following data analysis.

Action potential duration measurements. Optical action potential duration (APD) was measured at 80% repolarization (15, 37), and two-dimensional (2D) APD maps were constructed to reveal the spatial distribution of APDs on both LV and RV epicardial surfaces of the heart. Action potential (AP) traces have been normalized to peak values for representation purposes only. Mean APD was obtained at different BCLs by averaging APDs from all pixels. For presentation purposes only, 2D APD maps from BCL = 190 ms are shown in Figs. 2–5.

Conduction velocity measurements. Local conduction velocity (CV) was calculated as described previously (6, 33). Specifically, the distributions of activation times [measured at (dV/dt)max (44) for the spatial regions of 3 × 3 pixels were fitted with the plane, and gradients of activation times gₓ and gᵧ were calculated for each plane along the x- and y-axes, respectively. The magnitude of the local CV was calculated for each pixel as (gₓ² + gᵧ²)⁻¹/². Mean values for CV were calculated for the visible RV and LV surfaces. The relative change in CV compared with control was calculated for the RV and LV separately as follows:

\[ \Delta CV_{\text{Condition}} = \frac{CV_{\text{Condition}} - CV_{\text{Control}}}{CV_{\text{Control}}} \]

where condition means FCCP, Gmide, or ischemia.

Intraventricular heterogeneity. The spatial dispersion of APD, in the RV and LV separately, was estimated based on the heterogeneity index, μ (33), for control and all conditions during ischemia:

\[ \mu = \frac{\text{APD}^{95} - \text{APD}^{5}}{\text{APD}^{50}} \]

where APD⁹⁵ and APD⁵ represent the 95th and 5th percentiles of the APD distribution, respectively, and APD⁵⁰ is the median APD distribution.

VF analysis. VF analysis was performed in the hearts pretreated with 50 nM FCCP with (n = 3) and without (n = 3) blebbistatin that went to sustained VF (>1 min) during ischemia. During sustained VF, optical movies were taken every 10 s, and the first 1,000 frames (1.7 s) of each episode were used for analysis. To reveal singularity points, we constructed phase maps as previously described (19, 27, 51). We applied fast-Fourier transform to each pixel to obtain the power spectrum and to determine the distribution of frequencies in the range of 5–35 Hz. The dominant frequency (DF) was defined as the frequency corresponding to the highest peak in the power spectrum (7). We constructed 2D DF maps and used them to determine the mean DF separately in the RV and LV. To analyze the consecutive positions of the excitation wavefront, time-space plots (TSPs) were constructed as described previously (38, 39, 45).

Statistical Analysis

APD, CV, and DF data are presented as means ± SE. Statistical comparisons between control and different conditions in the same heart (ischemia, Gmide, FCCP, or RP) were performed using a two-sample Student’s t-test; RV and LV comparisons were performed using a paired Student’s t-test. Fisher’s exact test. Data in Table 2 were analyzed using one-way ANOVA with a Bonferroni correction. Values of P < 0.05 were considered statistically significant. F values <0.05 were statistically significant.

RESULTS

Table 1 illustrates the number of hearts that went into a sustained (>1 min) ventricular arrhythmia (ventricular tachy-
Table 1. No. of hearts that went into ventricular tachycardia/fibrillation

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>Ischemia</th>
<th>RP</th>
<th>Total Experiments</th>
<th>P Value Ischemia</th>
<th>P Value RP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemia with blebbistatin</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemia without blebbistatin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FCCP (100 nM)</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0.003‡</td>
<td>0.061</td>
</tr>
<tr>
<td>FCCP (50 nM) with blebbistatin</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.008‡</td>
<td>0.167</td>
</tr>
<tr>
<td>FCCP (50 nM) without blebbistatin</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.008‡</td>
<td>0.167</td>
</tr>
<tr>
<td>FCCP (30 nM)</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>0.200</td>
<td>0.310</td>
</tr>
<tr>
<td>Gmide (10 µM)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gmide + FCCP (30 nM)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RP, reperfusion; FCCP, carbonyl cyanide p-(tri-fluoromethoxy)phenyl-hydrazone; Gmide, glibenclamide. ‡P < 0.05 was considered significant.

cardia (VT) or VF]. During ischemia alone with blebbistatin, zero hearts out of seven went into VT/VF; however, during RP, two out of these seven hearts went into VF. During ischemia alone without blebbistatin (n = 2), no hearts went into VF. After 100 nM FCCP pretreatment (n = 4), or 50 nM FCCP with (n = 3) or without (n = 3) blebbistatin, all hearts went into VF during ischemia. However, after 30 nM FCCP pretreatment, only three out of eight hearts went into VT/VF during ischemia and an additional two hearts went into VF during RP. No hearts treated with 10 µM Gmide alone (0/3) or in combination with FCCP (0/3) went into sustained VF during ischemia or RP.

We first investigated the effects of no-flow global ischemia alone on APD in the rabbit heart. Figure 2A shows a field of view from our cameras and a representative example of a 2D APD map of control, 15 min of ischemia, and RP during pacing at BCL = 190 ms, and Fig. 2B shows representative traces of APs from the pixels marked as an asterisk in Fig. 2A. During control, the RV and LV have similar APD [134.4 ± 5.4 and 130.5 ± 4.5 ms, P = not significant (NS)]; however, ischemia leads to a reduction of APD in both the RV and LV (104.9 ± 8.4 and 101.9 ± 4.9 ms, respectively; P < 0.05 with control). RP restores APD in both ventricles (RV: 133.8 ± 6.9 ms, LV: 128.3 ± 9.0 ms; P = NS with control). The mean values of APDs for BCL=190 ms from all experiments (n = 5) are shown in Fig. 2C, separately for the RV and LV. Figure 2D represents mean APDs from different BCLs for control and ischemia from all experiments, in the RV and LV separately. Note the significant reduction of APD during ischemia in both ventricles at all BCLs.

Because all hearts went into VF during 100 and 50 nM of FCCP (see Table 1), we reduced the concentration of FCCP to 30 nM. Figure 3A illustrates representative 2D APD maps for control, FCCP, 15 min of ischemia, and RP conditions at BCL=190 ms, and representative traces of APs from single pixels denoted by the asterisks are shown in Fig. 3B. The mean values of APD from BCL=190 ms from all experiments (n = 3) are shown in Fig. 3C. FCCP (30 nM) does not change the APD in the RV (142.0 ± 7.3 vs. 139.7 ± 3.1 ms in control, P = NS) and slightly decreases it in the LV (122.9 ± 20.5 vs. 137.2 ± 0.9 ms in control, P = NS). Ischemia shortened APD in both RV (119.6 ± 6.3 ms, P = NS with control) and LV (89.2 ± 9.9 ms, P < 0.05 with control). Note that this effect is significant only for the LV, suggesting the presence of interventricular heterogeneity. APD returns to control conditions upon RP (RV: 127.9 ± 8.3 ms, P = NS with control; LV: 122.0 ± 18.4 ms, P = NS with control). Mean APDs for different values of BCLs for control and ischemia after FCCP pretreatment are shown in Fig. 3D for the RV and LV separately. While ischemia and FCCP decrease APD for both the RV and LV at all BCLs, the effect is significant (P < 0.05) only in the LV. Note that three out of eight hearts treated with 30 nM of FCCP went into VF during ischemia (Table 1). Therefore, these results indicate that depolarizing the mitochondria using small concentrations of FCCP is arrhythmogenic in the heart, in contrast to isolated cells (8).

To determine whether increased interventricular APD heterogeneity plays a role in VF initiation, we treated hearts with Gmide, which is known to abolish RV-LV heterogeneity (15, 34). Figure 4A represents typical examples of 2D APD maps for control, Gmide (10 µM), ischemia, and RP at BCL=190 ms, and representative traces of APs from single pixels denoted by the asterisks are shown in Fig. 4B. The mean values of APD for BCL=190 ms from all experiments (n = 3) are shown in Fig. 4C. Gmide leads to a prolongation in APD both in the RV (135.7 ± 4.2 vs. 127.9 ± 4.2 ms, P < 0.05) and LV (135.6 ± 4.5 vs. 128.5 ± 2.1 ms, P = NS). In contrast to untreated hearts (see Fig. 2), ischemia does not induce a significant decrease of APD either in RV (115.1 ± 19.0 ms, P = NS with control) or LV (129.3 ± 9.4 ms, P = NS with control), and the effect of RP is also mild. Figure 4D represents mean APDs for different BCLs for control and ischemia after Gmide pretreatment from all experiments, in the RV and LV separately. Note the insignificant change of APD during ischemia in both RV and LV. These results indicate that Gmide protects against the APD reduction and induction of interventricular heterogeneity that is

Table 2. RV-LV APD differences between ischemia and ischemia with condition

<table>
<thead>
<tr>
<th>Condition</th>
<th>300</th>
<th>260</th>
<th>220</th>
<th>190</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemia</td>
<td>20.64 ± 9.88</td>
<td>18.72 ± 4.87</td>
<td>11.97 ± 3.62</td>
<td>3.00 ± 5.87</td>
</tr>
<tr>
<td>FCCP (30 nM) + ischemia</td>
<td>35.63 ± 19.80</td>
<td>24.22 ± 14.28$</td>
<td>21.99 ± 2.78$</td>
<td>30.34 ± 6.11$</td>
</tr>
<tr>
<td>Gmide + ischemia</td>
<td>3.10 ± 1.57</td>
<td>3.30 ± 1.84</td>
<td>8.18 ± 0.05</td>
<td>1.24 ± 1.74</td>
</tr>
</tbody>
</table>

RV, right ventricle; LV, left ventricle; BCL, basic cycle length. $P < 0.05 was considered significant.
seen in ischemia alone (Fig. 2D). Note that no hearts treated with 10 μM Gmide went into sustained VF (see Table 1); therefore, pretreatment with Gmide is antiarrhythmic.

To determine if Gmide would still abolish interventricular heterogeneity if the mitochondrial network was depolarized, we performed similar experiments using 30 nM FCCP. Figure 5A represents typical examples of 2D APD maps for control, Gmide (10 μM), FCCP (30 nM), ischemia, and RP at BCL = 190 ms, and representative traces of APs from single pixels denoted by the asterisks are shown in Fig. 5B. The mean values of APD for BCL = 190 ms from all experiments (n = 3) are shown in Fig. 5C. Neither Gmide nor FCCP affect APD compared with control both in the RV (control: 137.3 ± 1.9 ms; Gmide: 137.1 ± 2.1 ms, P = NS, FCCP: 138.5 ± 4.3 ms, P = NS) and LV (control: 136.0 ± 2.3 ms; Gmide: 142.1 ± 5.0 ms, P = NS, FCCP: 141.1 ± 4.1 ms, P = NS). Ischemia induces a slight reduction of APD in both the RV (108.8 ± 9.3 ms, P = NS with control) and LV (123.6 ± 2.8 ms, P = NS with control), which was less than during ischemia alone and was abolished upon RP (RV: 131.4 ± 11.9 ms, LV: 141.4 ± 5.5 ms, P = NS with control). Figure 5D represents mean values of APD for different BCLs for control and ischemia from all experiments separately for the RV and LV. Note that, in contrast to Fig. 4D, addition of FCCP abolished the effect of Gmide and leads to a reduction of APD in the heart during ischemia. Nevertheless, this reduction is not significant compared with control, at all BCLs, in contrast to FCCP alone (Fig. 3D). However, no hearts went into VF during ischemia or RP (see Table 1), which suggests that the abolishment of interventricular heterogeneity provides the antiarrhythmic effect in the whole rabbit heart.

The presence of interventricular heterogeneity in the heart during different ischemic conditions is summarized in Fig. 6, where mean APDs from RV and LV are compared at different BCLs. Figure 6A indicates that, during ischemia alone, there is a significant difference (P < 0.05) between RV and LV at some BCLs (260 and 220 ms). However, this interventricular heterogeneity is increased and present at all BCLs when the
heart was pretreated with 30 nM FCCP (Fig. 6B). In contrast, pretreatment with Gmide and Gmide with FCCP (Fig. 6, C and D, respectively) reduces interventricular APD heterogeneity. Therefore, our data indicate that an increase in interventricular heterogeneity provides a possible substrate for arrhythmogeneity in the heart pretreated with FCCP during ischemia.

Table 2 illustrates statistical comparisons between interventricular heterogeneity during ischemia to the one during ischemia with different conditions, at different BCLs. At all BCLs during ischemia after pretreatment with 30 nM FCCP, the interventricular heterogeneity is significantly higher compared with ischemia alone. On the other hand, there is no significant difference in interventricular heterogeneity during ischemia after pretreatment with either Gmide or Gmide with 30 nM FCCP. Therefore, our results indicate that 30 nM FCCP pretreatment introduces interventricular APD heterogeneity during ischemia, which is significantly larger than during ischemia alone.

We also investigated how depolarizing the mitochondrial network affects other electrophysiological parameters in the heart during ischemia. Figure 7 illustrates the change of CV compared with control conditions during ischemia after pretreatment with 30 nM FCCP and/or Gmide for the RV (Fig. 7A) and LV (Fig. 7B) separately. Note the reduction of CV in both the RV and LV during all conditions. However, pretreatment with 30 nM FCCP introduces a significant difference ($P < 0.05$) between the RV and LV CV, thus leading to interventricular heterogeneity in propagation. Moreover, pretreatment with Gmide abolishes this interventricular CV heterogeneity ($P = NS$). Therefore, depolarizing the mitochondrial network not only introduces interventricular APD but also CV heterogeneity.

To examine the spatial dispersion of APD in each ventricle, the heterogeneity index, $\mu$, was calculated for control and all conditions during ischemia as shown in Fig. 7, C and D, for the RV and
LV separately. During ischemia with all different conditions, $\mu$ was significantly increased in both the RV and LV compared with control. However, no differences between the heterogeneities within the RV and LV were observed at any condition.

To demonstrate that interventricular heterogeneity provides a direct substrate for wavebreaks, we analyzed episodes of VF that occurred during ischemia with 50 nM FCCP pretreatment, in the presence ($n = 3$) or absence ($n = 3$) of the electrome-

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**Fig. 6. Interventricular APD heterogeneity.** Mean APDs from different BCLs for RV (black) and LV (red) after ischemia alone (A), during ischemia after pretreatment with 30 nM FCCP (B), during ischemia after pretreatment with 10 $\mu$M Gmide (C), and during ischemia after pretreatment with both 10 $\mu$M and 30 nM FCCP (D). #Statistical significance ($P < 0.05$) between RV and LV.

**Fig. 7. Effects of the condition during ischemia on conduction velocity (CV) and the spatial dispersion of APD, $\mu$.** There is a CV reduction in the RV (A) and the LV (B). There is an increase in $\mu$ during all conditions during ischemia in the RV (C) and LV (D). *Statistical significance ($P < 0.05$) between condition and control. #Statistical significance ($P < 0.05$) between RV and LV.
chanical uncoupler blebbistatin. VF was induced in all hearts, independent of the presence of blebbistatin (see Table 1). Hearts were positioned on the anterior surface so that the camera captured both the RV and LV with a clearly marked RV-LV junction. Representative examples of phase movies and TSPs are presented in Fig. 8. In Fig. 8A, selected snapshots of phase movies at different times are shown without (left) and with (right) blebbistatin. Here, white dotted lines show the location of the RV-LV junction, and black solid lines show the sites where TSPs were taken. A representative TSP below the phase movies shows the propagation patterns of electrical activity along the \( x'-x \) line, across the RV-LV junction during 1 s of activity. The TSP reveals an intermittent phase of discontinuity across the RV-LV junction. Some of the wavefronts moving from the RV to the LV pass the border interrupted; others form a Christmas tree pattern characteristic of a spiral wave (39, 51); and others are completely blocked. Phase discontinuities observed in the TSP imply that there are breaking waves at the border between RV and LV. White circles (Fig. 8A) indicate the position of singularity points, corresponding to the tips of broken wavebreaks (19, 27, 51). Note that singularity points were found mostly at the RV-LV junction for all VF episodes. Figure 8B shows the mean DF in the RV and LV separately without (left) and with (right) blebbistatin from all our experiments, illustrating no difference in VF dynamics between ventricles. Note that our results also indicate that blebbistatin has minimal effect on a substrate for VF in the heart with depolarized mitochondria.

**DISCUSSION**

In this manuscript, we investigated the electrophysiological effect of low concentrations of FCCP on the heart’s dynamics. The main findings of this study are: 1) pretreatment with 100 or 50 nM FCCP, with or without blebbistatin, leads to VF during ischemia. 2) Wavebreaks during VF in ischemia after pretreatment with 50 nM FCCP occurred at the RV-LV junction. 3) Pretreatment with 30 nM FCCP significantly increases interventricular (but not intraventricular) APD and CV heterogeneity, thereby providing the substrate for VF. 4) Pretreatment with Gmide leads to a reduction in interventricular heterogeneity, leading to an antiarrhythmic effect. Administration of FCCP acts to reverse this effect. Therefore, pretreatment with FCCP at concentrations shown to be cardioprotective at the single cell are arrhythmogenic in the whole rabbit heart.

**The Role of Mitochondria in Cardioprotection and Arrhythmogenesis**

Cardioprotection is considered to be pre- or postconditioning that reduces the damage occurring during prolonged ischemia by first administering short bursts (2–5 min) of ischemia-reperfusion (14). Both clinically and experimentally, cardioprotection manifests itself as a decreased area of risk, preservation of mechanical function after ischemia-reperfusion, and the presence of antiarrhythmic effects (20, 29, 36, 48). On the other hand, antiarrhythmic effects influence arrhythmogenesis.

![Fig. 8. Effects of blebbistatin on ventricular fibrillation (VF) dynamics. A: snapshots of phase movies without (left) and with (right) blebbistatin. White circles show singularity points at the border between the RV and LV (white dotted line). The black line \( x'-x \) shows where time-space plots (TSPs) were taken. B: mean dominant frequency (DF) between RV and LV without (left) and with (right) blebbistatin.](image-url)
only; therefore, an effect can be antiarrhythmic and not cardioprotective.

Depolarizing the mitochondrial network has been implicated in protecting the heart during prolonged ischemia injury. Ischemia causes an immediate disturbance in mitochondrial function, which includes a drop in the mitochondrial membrane potential (4), thus leading to consumption rather than production of ATP. Moreover, the damaged mitochondria cannot efficiently transfer electrons through the electron chain, which increases reactive oxygen species production (4), further damaging mitochondrial proteins (4). Both depolarization of the membrane potential and increase in reactive oxygen species are the leading factors that cause an impairment of mitochondrial and cellular function, resulting in necrotic and apoptotic cell death during prolonged ischemia (>30 min) (2). On the other hand, reactive oxygen species production transmits a signal to specific protective kinases and other mitochondrial kinases that lead to a cardioprotection mechanism if ischemia is prolonged (12).

Studies in single rat myocytes showed that treatment with low concentrations of FCCP (≤100 nM) improved posts ischemic recovery (8, 9) and thus is cardioprotective. In the single myocyte studies, cardioprotection was measured by examining mitochondrial oxidation and mitochondrial membrane potential, whereas in the whole heart, protection was determined by left ventricular end diastolic pressure (8, 9). However, our results demonstrate that FCCP changes electrophysiological properties of the heart, and administration of 100, 50, or 30 nM FCCP before no-flow global ischemia causes the heart to go into VF due to an increase in interventricular heterogeneity that cannot be evaluated in single cells. Therefore, in the whole heart, treatment with FCCP is arrhythmogenic.

**Interventricular Heterogeneity and Arrhythmogenesis**

During myocardial ischemia, there are several electrophysiological changes that make the heart vulnerable to VF (41, 50). One such change is decreased excitability (40, 41), which leads to a slowing of velocity of impulse propagation. If this reduction in conduction is not homogeneous within the ischemic tissue, it increases the likelihood for unidirectional conduction block and reentry (40). In our study, we found that CV is reduced during all conditions in ischemia. However, the interventricular difference in CV was only significant after depolarizing the mitochondrial network with FCCP. It has been shown experimentally that chamber-specific differences in the electrophysiological properties of the heart may represent a possible mechanism for the initiation and maintenance of ventricular arrhythmias (37, 40).

Pretreatment with FCCP induced interventricular heterogeneity in APD, thus providing an additional substrate for VF initiation. Indeed, during no-flow global ischemia alone (Figs. 2 and 6), despite the presence of interventricular APD heterogeneity, the heart never went into VF. These data are consistent with previous data in the whole rabbit heart that explored interventricular heterogeneity in APD and VF dynamics during ischemia (32). However, the interventricular APD heterogeneity increased during 30 nM FCCP pretreatment (see Figs. 3 and 6), thus providing an additional substrate for VF. To directly demonstrate the depolarization of the mitochondrial network provides the substrate for arrhythmogenesis, we examined VF dynamics (Fig. 8) after pretreatment with 50 nM FCCP. Our results reveal that singularity points are located at the RV-LV junction, and TSPs show an intermittent phase of discontinuity across the RV-LV junction.

In contrast, during pretreatment with 10 μM Gmide, interventricular APD and CV heterogeneity was absent, and hearts never developed VF, implying that indeed the presence of interventricular heterogeneity is a possible substrate for ischemia-induced arrhythmias. Similarly, a recent study by Morita et al. (34) found that Gmide abolished the asymmetric transseptal conduction that occurred during ischemia.

In addition, the single cell studies (8, 9) did not distinguish between RV and LV myocytes; therefore, we cannot directly compare our RV and LV data with the single cell data. Moreover, the ionic mechanisms of FCCP between the RV and LV may be different. Indeed, studies have shown that there are interventricular differences in the ionic regulations in RV and LV myocytes in different animals, including rabbits (37), which may play a role in this mechanism. On the other hand, it is also possible that there are differences in the density of mitochondria between the RV and LV, which could be a plausible explanation between the differences in FCCP action.

It should be noted that VF was not observed during control global ischemia (Table 1) similar to our previous study (32). However, several studies in the rabbit (30, 46, 47), pig (11, 22, 25, 42), human (31), and rat (13, 17, 18) found thatVF could be induced in the heart during ischemia [see also the excellent review by Janse and Wit (23)]. In the studies in the rabbit heart (30, 46, 47),VF was induced by burst pacing, which differs from our study whereVF arose spontaneously from our pacing protocol after pretreatment with FCCP.

**The Role of ATP-Dependent K⁺ Channels as a Possible Ionic Mechanism in Arrhythmogenesis**

Our results indicate that interventricular APD and CV heterogeneity may provide the substrate for arrhythmogenesis when the mitochondrial network was depolarized; however, we did not investigate the exact ionic or mechanical mechanisms of depolarizing the mitochondrial network. One of the possible mechanisms involves ATP-sensitive K⁺ (K_{ATP}) channels, which have been shown to play a major role in cardioprotection (12, 35). During ischemia, K_{ATP} channels are opened due to the decrease in ATP concentration, leading to an elevation in extracellular K⁺. It has been suggested experimentally that all K_{ATP} channel openers are cardioprotective (20, 29, 48) since they promote repolarization and a shortening of APD due to the increase in extracellular K⁺. On the other hand, it has been suggested that K_{ATP} channel blockers, such as Gmide, do not protect the heart during ischemic injury and will actually make the ischemic area larger (20). However, an optical mapping study by Morita et al. (34), which used Gmide to assess the transmural differences in the canine interventricular septum during ischemia, found, similar to our results, the effect of Gmide was antiarrhythmic and abolishes interventricular heterogeneity, suggesting that K_{ATP} channels mediate its effects on the extracellular K⁺ and reduces the dispersion of repolarization. Moreover, a recent optical mapping study involving nonfailing and failing human myocardium showed that Gmide was able to terminate VF induced from treatment with K_{ATP} openers, thus providing further evidence of the antiarrhythmic effect of Gmide (15). Even though it has been shown that K_{ATP}...
blocks are not cardioprotective, they still mediate an important antiarrhythmic effect during ischemic injury. Although $K_{\text{ATP}}$ channels can be considered to be a good candidate to reveal the ionic mechanism of cardioprotection, we cannot rule out the fact that FCCP may partially act on gap junctions or provide other methods of myocyte desynchronization. While the ionic mechanisms are important and need to be examined in the cardioprotective or antiarrhythmic effects, it was beyond the scope of our study.

Moreover, it is important to note that application of FCCP before the ischemic period is expected to decrease the free energy change of ATP hydrolysis. Only by combining this condition with the moderate decrease of ATP during ischemia in the absence of any mechanical load will decrease the free energy change to a level where opening of the ATP-sensitive channels becomes manifest. Therefore, it would be required to analyze ATP determinants, whereas analysis of the gradient in APD would require channel density measurements. While these analyses are important to understand the exact mechanism of how FCCP affects ATP concentration and the density of ATP-sensitive channels, it was beyond the scope of our study to investigate the exact mechanism of such action. However, in future studies, this should be examined.

The Role of Blebbistatin During Arrhythmogenesis

To eliminate motion artifacts during optical mapping experiments, a small concentration of blebbistatin (10–15 μM) was used. Blebbistatin blocks cardiac contractions without affecting electrical activity, including ECG parameters, atrial and ventricular effective refractory periods, and atrial and ventricular activation patterns (16). However, a recent study by Lou et al. (28) showed that, in the rabbit, the incidence of arrhythmia was significantly lower than with the use of other electromechanical uncouplers. It has to be noted that blebbistatin is a myosin ATPase inhibitor that liberates a significant amount of ATP for sarcomeric consumption. Therefore, responses of the heart to ischemia might be affected. To confirm that blebbistatin does not affect VF inducibility in the heart during ischemia, we performed additional experiments without the use of blebbistatin during ischemia alone and found that the heart did not go into VF (see Table 1). Also, our additional experiments without the use of blebbistatin demonstrate that, when FCCP was added, the heart still went into VF (Fig. 8). Therefore, we are confident that VF is caused by FCCP and not from the use of blebbistatin.

In conclusion, FCCP is arrhythmogenic at low concentrations in the whole rabbit heart. A possible substrate for arrhythmias that develop due to mitochondrial uncoupling is the presence of interventricular APD and CV heterogeneity. In contrast, Gmide reduces interventricular heterogeneity that exerts an antiarrhythmic effect. Therefore, we suggest that uncoupling the mitochondria alone may provide the substrate for ischemia-induced arrhythmias.

Limitations

We investigated 20 min of no-flow global ischemia, which did not induce irreversible damage to the heart (10). We also perfused FCCP for 5 min, similar to Refs. 8 and 9. However, it is possible that the cardioprotective effect of FCCP could be seen during longer perfusion times or ischemia duration.

After our experiments, we performed TTC staining to ensure that our preparation was still viable after RP. However, there is a possibility that there is a potential difference in the level of perfusion between the LV and RV associated with the retrograde Langendorff perfusion system.

In this study, we used a small amount of the electromechanical uncoupler blebbistatin. Contraction determines a major part of the cardiac energy consumption (ATP consumption) during ischemia. This will not only affect the rate of ATP decrease but also the rate of the development of metabolic and respiratory acidosis (in the case of ischemia, CO$_2$ trapping). Both changes will have a major effect on ischemic depolarization, extracellular $K^+$ accumulation, and APD shortening. Therefore, blebbistatin, which decreases energy consumption, can exert a marked effect on the electrical changes during acute ischemia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: R.M.S. and S.S.V. performed experiments; R.M.S. and E.G.T. analyzed data; R.M.S. and E.G.T. drafted manuscript; E.G.T. conceived and designed the research; E.G.T. interpreted results of experiments; E.G.T. edited and revised manuscript; E.G.T. approved the final version of manuscript.

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