Mitochondrial uncoupling, with low concentration FCCP, induces ROS-dependent cardioprotection independent of $K_{\text{ATP}}$ channel activation

Jonathan P. Brennan$^a$, Richard Southworth$^b$, Rodolfo A. Medina$^{b,1}$, Sean M. Davidson$^c$, Michael R. Duchen$^d$, Michael J. Shattock$^a$,*

$^a$Cardiac Physiology (Cardiovascular Division), The Rayne Institute, St Thomas’ Hospital, King’s College London, SE1 7EH, UK
$^b$NMR Laboratory (Division of Imaging Sciences), King’s College London, SE1 9RT, UK
$^c$The Hatter Cardiovascular Institute, University College London, WC1E 6HX, UK
$^d$Mitochondrial Biology Group (Department of Physiology), University College London, WC1E 6BT, UK

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Abstract

Objective: Both $K_{\text{ATP}}$ channel opening drugs and ischaemic preconditioning have been suggested to protect the ischaemic heart by acting on $K_{\text{ATP}}$ channels in the inner mitochondrial membrane, uncoupling the proton gradient and partially dissipating the mitochondrial membrane potential. The aim of these studies was to use low concentrations of FCCP, a mitochondrial protonophore, to bypass the mitochondrial $K_{\text{ATP}}$ channel and partially uncouple the mitochondria and establish whether this activates protective pathways within the rat heart analogous to $K_{\text{ATP}}$ channel openers or preconditioning.

Methods: Isolated, Langendorff-perfused rat hearts were subjected to 25 min global zero-flow ischaemia and functional recovery assessed. Hearts were pretreated with FCCP (30–300 nM) in the presence or absence of glibenclamide (1 $\mu$M), 5-hydroxydecanoate (5-HD; 100 $\mu$M), N-acetyl cysteine (4 mM), or N-2-mercaptopropionyl glycine (1 mM). The metabolic consequences of FCCP perfusion in isolated hearts were studied using $^{31}$P NMR, and reactive oxygen species (ROS) production was measured using DCF fluorescence in isolated rat ventricular myocytes.

Results: FCCP exerted a dose-dependent cardioprotective effect, with 100 nM FCCP being the optimal concentration. This effect could not be blocked by glibenclamide or 5-HD, but was completely attenuated by N-acetyl cysteine and N-2-mercaptopropionyl glycine. Perfusion with FCCP (100 nM) did not deplete bulk ATP during the pretreatment period but significantly depleted phosphocreatine. In ventricular myocytes, FCCP caused an antioxidant-sensitive increase in ROS production but diazoxide was without effect.

Conclusions: In the isolated rat heart, partial mitochondrial uncoupling with low-dose FCCP significantly improves post-ischaemic functional recovery via a ROS-dependent pathway. This cardioprotection is not mediated via the depletion of cellular ATP or mitochondrial $K_{\text{ATP}}$ channel activation.

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1. Introduction

Both the signalling pathway and the ‘end effector’ underlying the cardioprotective phenomenon of ischaemic preconditioning remain uncertain. However, a number of studies have implicated the ATP-sensitive potassium channels of the inner mitochondrial membrane ($m$-$K_{\text{ATP}}$) either as an end effector or, more recently, as simply a link in a complex signalling cascade (for review see [1]). It has been proposed that opening of the $m$-$K_{\text{ATP}}$ channel, prior to index ischaemia, during preconditioning cycles, or with pharmacological agents such as diazoxide, can act as a trigger and confer protection from a subsequent ischaemic [2,3].
Opening the \( \text{mitoK}_{\text{ATP}} \) channel may result in a partial depolarisation of the mitochondria, either as a direct result of \( K^+ \) entry or by activation of \( K^+/H^+ \) exchanger in response to the \( K^+ \) entry. It is suggested that this depolarisation may reduce mitochondrial \( Ca^{2+} \) entry, reducing mitochondrial \( Ca^{2+} \) overload and related damage [4], or may activate protective signalling pathways by increasing mitochondrial reactive oxygen species (ROS) production, which lead to activation of downstream end-effectors [5]. Many studies have shown ROS to play a significant role in both ischaemic preconditioning and diazoxide-induced cardioprotection. Exogenous application of oxidants induces preconditioning in intact hearts [6] and antioxidants block the beneficial effects of preconditioning in a variety of models [3,7–9]. However, the degree of mitochondrial depolarisation that occurs during preconditioning and whether this, itself, leads to increased ROS production is not yet clear. Liu and colleagues have shown that diazoxide causes an increase in flavoprotein fluorescence [10]. However, while flavoprotein oxidation has been suggested to be closely linked to mitochondrial membrane potential (\( \Delta \Psi \)), it is unclear from these studies if, or by how much, the mitochondria are depolarised by such preconditioning mimetics.

Holmuhamedov et al. [11] have shown that \( \text{mitoK}_{\text{ATP}} \) channel openers pinacidil, cromakalim, and levcromakalim all induce concentration-dependent mitochondrial membrane depolarisation, in isolated rat heart mitochondria. They also demonstrated that \( K_{\text{ATP}} \) channel openers diazoxide and pinacidil induced mitochondrial membrane depolarisation and limited \( Ca^{2+} \) uptake by isolated mitochondria [4]. In addition, Minners et al. have shown mitochondrial depolarisation in two cell lines in response to ischaemia, adenosine and diazoxide treatment [12]. However, it has been suggested that opening of the \( \text{mitoK}_{\text{ATP}} \) channel either during preconditioning or with \( K^+ \) channel openers would only depolarise the mitochondria by 1–2 mV [13]; changes too small to affect \( Ca^{2+} \) handling by the mitochondria or mitochondrial energetics. In addition to these observations there are studies demonstrating that direct mitochondrial uncoupling interventions can also induce cardioprotection. Data from Minners et al. [14] and Rodrigo et al. [15] have demonstrated cardioprotection with transient administration of 2,4-dinitrophenol (DNP).

Whether low concentrations of mitochondrial uncouplers induce protection via analogous mechanisms to those described for \( \text{mitoK}_{\text{ATP}} \) channel opening, however, remains to be determined. In theory, introduction of a protonophore may have a similar effect to opening \( K^+ \) channels in the mitochondria; reducing \( \Delta \Psi \) and thus the driving force for ATP synthesis across the inner mitochondrial membrane. However, if, as Kowaltowski et al. suggest [13], only a very small depolarisation occurs with \( \text{mitoK}_{\text{ATP}} \) channel opening, perhaps only low concentrations of mitochondrial uncouplers may induce protection while high concentrations are likely to be detrimental. It is plausible that in the intact heart, \( \text{mitoK}_{\text{ATP}} \) channel openers induce only a very mild mitochondrial uncoupling, which can be compensated for by changes in mitochondrial metabolism without significant mitochondrial depolarisation.

Carbonyl cyanide \( p-(\text{tri-fluoromethoxy})\text{phenyl-hydrazone} \) (FCCP) is a potent uncoupler of oxidative phosphorylation in mitochondria [16]. It acts by collapsing the proton gradient [17,18] across the inner mitochondrial membrane, reducing the drive for ATP synthesis [19–23] and it has been demonstrated that –CCP uncouplers have a high-affinity binding site in rat heart mitochondria [24]. Much of the knowledge of the effects of FCCP in \( \text{in vivo} \) and \( \text{in vitro} \) is based on studies using concentrations above 1 \( \mu \)M, and submicromolar effects have yet to be characterised. Using low concentrations of FCCP it may be possible to bypass opening of the \( \text{mitoK}_{\text{ATP}} \) channels but induce a similar mild uncoupling of the mitochondria as is suggested to happen in \( \text{mitoK}_{\text{ATP}} \) channel-induced and ischaemic preconditioning-induced cardioprotection. If this is the case, it may be possible to use FCCP as a pharmacological tool to investigate the importance of mitochondrial uncoupling in cardioprotection.

2. Methods

2.1. Crystalloid perfusion of the rat heart

Male Wistar rats (250–300 g) were used in all studies. The investigation conforms with the \textit{Guide for the Care and Use of Laboratory Animals} published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Hearts were perfused in the Langendorff mode with modified Krebs–Henselit Buffer (K-HB) consisting of 118.5 mM NaCl, 25.0 mM NaHCO\(_3\), 4.75 mM KCl, 1.18 mM KH\(_2\)PO\(_4\), 1.19 mM MgSO\(_4\), 11.0 mM D-glucose, and 1.4 mM CaCl\(_2\). Left ventricular developed pressure was measured using a fluid-filled balloon. Hearts were paced at 360 bpm (at 1.5× the threshold voltage) until 2 min into ischaemia and pacing was resumed 10 min into reperfusion.

2.1.1. Protocols

The protocols used in these studies are shown in Fig. 1. The basic protocol involved a 30 min stabilisation period at constant perfusion pressure (73 mm Hg) followed by 5 min pretreatment, or vehicle control, with perfusion at constant flow (set to the flow at the end of the stabilisation period). This was followed by a 25 min period of zero-flow global ischaemia and 40 min of aerobic reperfusion at a constant pressure. In Protocols 2, 3 and 5 (see Fig. 1), the pretreatment protocol was preceded by 5 min of perfusion with either a \( K_{\text{ATP}} \) channel blocker (glibenclamide—1 \( \mu \)M or 5-hydroxydecanoate—100 \( \mu \)M) or an antioxidant (\( N\)-acetyl cysteine—4 mM or \( N\)-2-mercaptopropionyl glycine—1 mM) that was also present throughout the pretreatment period. There is no wash out period prior to the index ischaemia.

2.2. \( ^{31}P \) NMR spectroscopy

Isolated rat hearts (6 per group) were Langendorff perfused (37 °C) as per Protocol 1 (see Fig. 1) in the bore of an NMR magnet, immersed in coronary effluent throughout.
The pretreatment period was 5 min FCCP (100 nM) (constant flow) and control hearts were pretreated with the vehicle (0.0002% DMSO).

$^3$P NMR spectra were obtained at 162 MHz using a Bruker AM-400 wide-bore spectrometer as described previously [25]. All peaks were referenced to phosphocreatine (PCr) at $-2.52$ ppm (orthophosphoric acid at 0 ppm). Partially relaxed spectra (70° pulse, relaxation delay 1 s) were obtained, 48 scans per spectrum. Free Induction Decay signals (FIDs) were processed with a line broadening of 20 Hz prior to Fourier transformation. Quantification of PCr, Pi and ATP (from the $\beta$-ATP) was performed using Bruker software after first scaling all the spectra to a reference spectrum, and expressed in arbitrary units. Intracellular pH was calculated from the chemical shift difference between the Pi and PCr resonances with the equation from Bailey et al. [26]. Coronary effluent was collected at regular intervals and concentrations of lactate measured using an YSI 3200 Lactate Analyser.

### 2.3. Myocyte isolation

Ventricular myocytes were isolated from hearts of male Wistar rats (250–300 g) using a standard collagenase digestion protocol as described previously [27].

### 2.4. DCF fluorescence microscopy

An initial pilot study was undertaken to measure ROS production in response to a number of cardio-protective interventions. Cells were loaded with DCF, washed and stored until required. Using a Zeiss Axiosvert 200 microscope equipped with hardware and software (Metafluor v.5.0) from Universal Imaging Corporation (Downingtown PA), DCF was excited at 490 nM, and emitted light measured at 530 nM. Myocytes were perfused with the isolation buffer followed by buffer containing FCCP (100 nM), diazoxide (30 μM) or H$_2$O$_2$ (10 mM).

Following this, a further study was undertaken to confirm that FCCP-induced ROS production was sensitive to antioxidants. Cardiomyocytes were plated on laminin-coated coverslips for 1 h before use. The medium was replaced with imaging buffer (25 mM NaHCO$_3$, 116 mM NaCl, 5.4 mM KCl, 0.4 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 10 mM glucose, 20 mM taurine, 5 mM pyruvate, pH 7.4) containing 5 μM 2’,7’-dichlorofluorescin diacetate (DCF) (Molecular Probes). After 5 min the buffer was replaced with buffer alone or containing the following concentrations of compounds as indicated: 4 mM NAC, 1 mM MPG. Cells were then imaged on a Zeiss 510 CLSM confocal microscope equipped with ×40 oil immersion, quartz objective lens (numerical aperture 1.3). The cells were illuminated using the 488-nm emission line of an Argon laser set at low power to avoid inducing oxidation. Images were collected every 30 s, using a 505-nm long-pass filter. After 5 min, FCCP was added to a final concentration of 100 nM, and images were collected for a further 25 min. Images were analyzed by use of Zeiss software (LSM 2.8). The ratio of the final rate of fluorescence increase to the initial (before FCCP) rate was calculated and normalized to control wells with no addition of FCCP (normalized to 100).

### 2.5. Materials

FCCP was obtained from Sigma Chemical Co. Ltd. UK; stocks were made up in DMSO (0.5–150 mM) and then serially diluted to give final concentrations of 30–300 nM FCCP. The final DMSO concentration was constant throughout (0.0002%). Stock solutions of glibenclamide (Sigma
Chemical Co. Ltd. (UK) were dissolved in DMSO (0.01% in all groups) and diluted in K-HB. 5-HD (ICN, UK) stocks were dissolved in de-ionised water and diluted in K-HB. NAC and MPG were obtained from Sigma Chemical Co. UK. NAC (4 mM) was dissolved in K-HB as was MPG (1 mM). Diazoxide was obtained in aqueous saline solution (Goldshield Pharmaceuticals Ltd, Croydon, UK) and used at a final concentration of 30 μM.

2.6. Data analysis

Data are generally presented as means±S.E.M. unless stated otherwise. Data analysis used one-way ANOVA followed by a post-hoc Dunnett’s test to compare treatments to control, or post-hoc Student–Newman–Keuls test to compare between multiple groups. Where applicable, a repeated-measure ANOVA followed by Student–Newman–Keuls test was used to compare data over multiple time-points. Comparison between two groups was performed using unpaired Student’s t-test. In all cases P<0.05 was considered statistically significant.

3. Results

3.1. Does pretreatment with low dose FCCP protect the heart against ischaemia (Protocol 1)?

3.1.1. Effects of FCCP on baseline cardiac function

Hearts were perfused with FCCP (30–300 nM) for 5 min prior to a 25 min ischaemic period (Protocol 1). As shown in Table 1 there were no differences in coronary flow or LVDP between groups during the stabilisation period. In all cases FCCP caused a significant decline in perfusion pressure and LVDP by the end of the treatment period. 300 nM completely abolished all contractile function during the 5 min perfusion period, causing hearts to enter contracture prior to the ischaemic insult. This contracture was responsible for the relative increase in perfusion pressure in hearts treated with 300 nM (cf. those treated with 100 nM). In a separate series of experiments (not shown), the reversibility of 5 min infusion of FCCP was investigated in aerobically perfused hearts. The effects of 5 min perfusion with 100 nM FCCP were fully reversible with LVDP recovering to a value identical to the time-matched controls of 92±2% after 10 min washout.

3.1.2. Effects of FCCP on the post-ischaemic recovery of contractile function

Fig. 2 panels A and B show the relationship between the pretreatment concentration of FCCP and recovery of LVDP and LVEDP after ischaemia. This relationship was bell-shaped with a peak in protective efficacy after pretreatment with 100 nM FCCP. Lower concentrations of FCCP (1, 3 and 10 nM) were investigated in a pilot dose-ranging study and found to be without effect (data not shown). FCCP (100 nM) was very protective increasing recovery of LVDP from 14.7±1.9% in control hearts (n=8) to 57.8±5.1% in treated hearts. 300 nM FCCP conferred no protective effect on recovery following ischaemia (recovery=17.9±5.8%).

3.2. Is the FCCP-induced cardioprotection blocked by KATP channel antagonists (Protocol 2)?

Our starting hypothesis was that mild uncoupling of the mitochondria could provide cardioprotection independent of KATP channel activation. However, FCCP at high concentrations is a potent mitochondrial uncoupler [16] which will completely abolish oxidative metabolism and ATP synthesis [22,23]. It remains possible therefore that, at the cardioprotective concentration of FCCP (100 nM), energy metabolism may be compromised, ATP depleted and KATP channels activated. In order to determine whether KATP channel activation (subsequent to ATP depletion) played a role in the protection afforded by FCCP, hearts were perfused with KATP channel blockers before and during the FCCP pretreatment period (Fig. 1, Protocol 2).

The baseline and post-treatment (prior to ischaemia) haemodynamic data for hearts in Protocol 2 are also shown in Table 1. There were no differences between the experimental groups and their relevant controls for baseline coronary flow or LVDP. Glibenclamide caused a significant increase in perfusion pressure, presumably as it blocks KATP channels in vascular smooth muscle, which play an active role in vasodilation [28,29]. 5-HD is also shown to maintain perfusion pressure at levels significantly higher than control values after the treatment period, although this indicates an anomalous

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline contractile function and coronary flow measured in isolated hearts at the end of the stabilisation period and following pre-treatment (protocols shown in Fig. 1)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline CF (ml/min)</td>
</tr>
<tr>
<td>Control</td>
<td>11.5±0.6</td>
</tr>
<tr>
<td>FCCP 30 nM</td>
<td>11.9±0.5</td>
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<tr>
<td>FCCP 100 nM</td>
<td>11.8±0.3</td>
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<tr>
<td>FCCP 300 nM</td>
<td>11.8±0.5</td>
</tr>
<tr>
<td>Control</td>
<td>13.4±1</td>
</tr>
<tr>
<td>FCCP 100 nM</td>
<td>13.1±0.8</td>
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<tr>
<td>Glib 1 μM</td>
<td>13.2±0.5</td>
</tr>
<tr>
<td>FCCP+Glib</td>
<td>14.4±0.7</td>
</tr>
<tr>
<td>Control</td>
<td>12.8±0.4</td>
</tr>
<tr>
<td>FCCP 100 nM</td>
<td>13.5±0.4</td>
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<tr>
<td>5-HD 100 μM</td>
<td>14.2±0.5</td>
</tr>
<tr>
<td>FCCP+5-HD</td>
<td>13±0.3</td>
</tr>
<tr>
<td>Control</td>
<td>12.7±0.5</td>
</tr>
<tr>
<td>FCCP 100 nM</td>
<td>12.9±0.6</td>
</tr>
<tr>
<td>NAC 4 mM</td>
<td>12.4±0.7</td>
</tr>
<tr>
<td>FCCP+NAC</td>
<td>12.4±0.6</td>
</tr>
</tbody>
</table>

CF: coronary flow; LVDP: left ventricular developed pressure; PP: perfusion pressure; Glib: glibenclamide; 5-HD: 5-hydroxydecanoate; NAC: N-acetyl cysteine. Baseline perfusion was at a constant perfusion pressure of 73 mm Hg. During ‘Treatment’, perfusion was at a constant coronary flow set to a value equivalent to the prevailing flow at the end of the stabilisation period immediately prior to pretreatment. Hearts were then subjected to perfusion protocols as shown in Fig. 1. Data are mean±S.E.M. *P<0.05 vs. control.
result for the control group rather than indicating an effect of 5-HD. In the 5-HD limb of this study, it is unclear why perfusion pressure in the matched control group fell to 80±6%.

3.2.1. Effects of glibenclamide and 5-HD on the FCCP-induced increase in post-ischaemic recovery of contractile function

In Fig. 2 Panel C, as in Panel A, 100 nM FCCP pretreatment again induced significant improvement in LVDP recovery following ischaemia and reperfusion (46.5±4.9% from 30.8±3.9%). Glibenclamide (1 μM), a non-specific K<sub>ATP</sub> channel blocker—at a concentration greater than that previously shown to block diazoxide-induced protection [30], alone had no affect (28.8±6.1%) and when co-administered with FCCP did not block the improvement in LVDP recovery (52.7±3.6%). The protocol was repeated with the putative <i>mito</i>K<sub>ATP</sub> channel specific blocker, 5-HD (100 μM) (again at a concentration previously shown to block diazoxide-induced protection [30]). There was no significant difference between the protection afforded by FCCP and that following FCCP+5-HD (FCCP, 56.7±6.9% cf. FCCP+5-HD, 47.8±7.8) (Fig. 2D), although it was also noted that there is no significant difference between control and FCCP+5-HD. These results show that K<sub>ATP</sub> channel blockade was unable to completely reverse the FCCP-induced cardioprotection, and suggest that K<sub>ATP</sub> channel activation plays no significant role in this protection. However, in order to establish if 100 nM FCCP could limit oxidative phosphorylation to the extent that ATP concentrations were compromised, a series of studies were undertaken using NMR spectroscopy to measure high energy phosphate concentrations during the pretreatment protocol with 100 nM FCCP (Protocol 1).

3.3. Effect of a cardioprotective concentration of FCCP on metabolism

3.3.1. ATP during FCCP pretreatment and ischaemia

Fig. 3A shows ATP concentration measured using <sup>31</sup>P NMR spectroscopy throughout Protocol 1 (hearts pretreated with 100 nM FCCP). The total ATP concentration within hearts treated with FCCP (100 nM) did not decline during the 5 min pretreatment period. The ATP concentration in the FCCP treated group at the end of the 5 min pretreatment period
was 98±5% of the aerobic baseline. This was not significantly different from the ATP concentration in the time-matched control group. Thus, total ATP concentration is not depleted by the FCCP pretreatment and, unless sub-membrane or mitochondrial ATP concentration differs significantly from this total measurement, it is therefore unlikely that $K_{\text{ATP}}$ channels would be activated during this pretreatment period.

During ischaemia, however, ATP fell faster in the FCCP-treated hearts compared to control, giving an ATP concentration of 2±2% of baseline in FCCP treated hearts compared to 30±7% of baseline in control hearts at the end of ischaemia. This accelerated pattern of ATP depletion is similar to that reported in preconditioned isolated rat hearts by Kolocassides et al. [31], showing, at least, that similar conditions are present in FCCP-treated hearts to those previously observed in preconditioned hearts and that ischaemic ATP concentration is not a good determinant of post-ischaemic viability.

### 3.3.2. PCr and Pi during FCCP pretreatment and ischaemia

Phosphocreatine (PCr) decreased towards the end of the 5 min FCCP pretreatment period (to 76±8% baseline), indicating that, despite the maintenance of total ATP concentrations, energy metabolism may be compromised in hearts pretreated with 100 nM FCCP. The ratio of PCr to Pi, Fig. 3B, shows a similar pattern to that for PCr alone, indicating a significant metabolic compromise by the end of the 5 min FCCP pretreatment period compared to control hearts. This difference is maintained at all but one of the ischaemic time-points. Together these results indicate that, despite there being no decline in ATP during the pretreatment period, there is some degree of metabolic compromise occurring as indicated by the fall in the ratio of PCr/Pi. It is possible that the mild uncoupling effect of FCCP is challenging ATP synthesis, and for a short period of time the mitochondria can meet this challenge, before using up stores of ATP with the extra ischaemic challenge.

### 3.3.3. Lactate and $\text{pH}_i$ during FCCP pretreatment and ischaemia

Fig. 3C shows that the coronary lactate efflux did not rise during the 5 min pretreatment period, suggesting that there was no significant switch to glycolytic metabolism. Lactate, which accumulates intracellularly during ischaemia, was then washed out during the early minutes of reperfusion. The post-ischaemic washout of lactate was, however, similar in both control and FCCP-treated groups.

Fig. 3D shows intracellular pH ($\text{pH}_i$) measured throughout the protocol. There was no change in $\text{pH}_i$ during the pretreatment period in either of the groups; hearts only become acidotic once ischaemic. During ischaemia, $\text{pH}_i$ fell more rapidly in FCCP-treated hearts, although both control and FCCP-treated hearts reached similar $\text{pH}_i$ values by the end of the ischaemic period, in line with all endogenous glycogen being depleted at this time point in both groups.

### 3.4. The role of ROS in the cardioprotection

#### 3.4.1. Effect of an antioxidant on FCCP-induced cardioprotection

A separate series of experiments (Protocol 3) tested whether protection could be blocked by an antioxidant, as has been reported for preconditioning and diazoxide-induced

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Fig. 3. Metabolic consequences of FCCP pretreatment. Hearts were perfused according to Protocol 1 (Fig. 1) and subjected to pretreatment with FCCP (100 nM) prior to 25 min global ischaemia. ATP, PCr and Pi were measured using $^{31}$P NMR and coronary lactate as described. Mean values for [ATP] (A), PCr/Pi ratio (B) and coronary effluent lactate concentration (C), are expressed as a percentage of baseline. $\text{pH}_i$ (D) is expressed in absolute pH units. All values are expressed as means±S.E.M. ($n=6$/ group). *$P<0.05$ vs. control.
protection [32,33]. Table 1 shows that NAC did not effect the decline in LVDP during FCCP treatment, however it did partially block the decrease in perfusion pressure to a level that was no longer significant. This suggests that while the acute effects of FCCP on contractile function are not ROS related the effects on vascular tone and therefore perfusion pressure may at least be partially mediated by increased ROS.

3.4.1. Post-ischaemic contractile function. Fig. 2E shows functional recovery following reperfusion in the hearts treated with FCCP and NAC. In this series, FCCP improved LVDP recovery from 33.4±4.2% to 58±5.3% (n=6). NAC alone had no effect on functional recovery (30.5±4.8% cf. Control) but when co-administered with FCCP completely abolished the protective effect of FCCP, decreasing LVDP recovery to 14±5.5%. Similar results are shown in Fig. 2F, showing MPG (1 mM) also attenuated the FCCP-induced cardioprotection.

3.4.2. Measuring ROS production during cardioprotective interventions

Fig. 4A shows the influence of FCCP, in the absence and presence of antioxidants, on the rate of ROS production in isolated rat myocytes. Fig. 4B shows typical traces for each treatment. FCCP caused a significant increase in the rate of ROS production compared to control which was completely blocked by both NAC and MPG. FCCP increased the rate of ROS production by an average of 158% which was brought back to 95% with NAC pre-treatment and 29% with MPG pre-treatment.

Fig. 4C shows a separate series of experiments in which the increase in ROS production by FCCP is confirmed and compared to a positive control (H2O2) and diazoxide. FCCP induced a significant increase in the rate of ROS production but diazoxide failed to show a significant change.

4. Discussion

FCCP pretreatment significantly improved post-ischaemic functional recovery when administered prior to global ischaemia in the isolated rat heart. This is in agreement with the initial hypothesis, that a low-dose FCCP treatment would induce partial uncoupling of mitochondria and activate potentially protective signalling pathways. However, it is not possible to conclude from these data alone whether the pathways activated by FCCP have any similarities to those activated by preconditioning or diazoxide.

The relationship between the concentration of FCCP and cardioprotection was ‘bell-shaped’ with an optimal concentration at 100 nM, with 300 nM causing contractile failure. Thus, a small cardioprotective window exists in which mild uncoupling with FCCP is protective while any higher degree of uncoupling is deleterious. The protection afforded by 100 nM FCCP also appeared to be independent of KATP channel opening. Three lines of evidence support this contention:

(i) Protection was unaffected by KATP channel blockade with either glibenclamide or 5-HD.
(ii) Protection occurred without a detectable fall in intracellular ATP content.
(iii) 100 nM FCCP induced mitochondrial flavoprotein oxidation without activating sarcolemmal KATP channels (see following paper [34]).
Thus, it is unlikely that FCCP acts by indirectly opening \( K\text{ATP} \) channels but rather by ‘short-circuiting’ them and introducing a parallel leak which would tend to dissipate the mitochondrial proton gradient.

The decline in PCr/P\(_i\) ratio towards the end of the pretreatment period suggests that FCCP-pretreated hearts were metabolically compromised, and this may manifest itself as the reduced cardiac function seen during FCCP-treatment. However this was not sufficient to deplete bulk ATP during this 5 min period or to induce a significant switch to anaerobic glycolysis (as would be evidenced by increased lactate production). As we measure bulk ATP it remains possible that transmural ATP gradients may exist, however these are not significant enough to alter bulk ATP in FCCP treated hearts compared to control. In addition it is unlikely that even the largest transmural ATP gradient could account for the increased ATP depletion of FCCP treated hearts during ischaemia compared to control hearts.

A low concentration of a protonophore such as FCCP will tend to dissipate the mitochondrial proton gradient and hence reduce the driving force for ATP production. However, at the cardioprotective concentration studied (100 nM), it appears that the myocytes are either able to oppose this by increasing electron transport and proton efflux or can buffer the changes in ATP by depleting PCr. However, once ischaemic, the preceding depletion of the PCr stores by FCCP accelerates the ischaemia-induced decline in ATP. The accelerated profile of ATP decline in ischaemia is similar to that described in preconditioned rat hearts [35,36], and while this may be coincidental it is clear that both FCCP and preconditioning can achieve protection despite an accelerated fall in ATP. Alternatively, it has also been proposed that mild mitochondrial uncoupling can enhance ATP production, during the index ischaemia, either by partially uncoupling the mito- chondria [37] or by causing mitochondrial swelling [38]. However, our results suggest that in FCCP-protected hearts it is unlikely that ATP synthesis is maintained or enhanced as ATP levels fall more quickly during index ischaemia.

The protection afforded by FCCP pre-treatment in isolated hearts, and the ROS production in isolated cells, was abolished by the co-administration of the antioxidants NAC and MPG. Baines and colleagues have suggested that dissipation of the proton gradient by \( K\text{ATP} \) channel openers and mitochondrial depolarisation leads to an increase in oxidant stress [32]. This oxidant stress in turn activates pathways down-stream from the mitochondria activating an eventual unidentified “end-effec- tor”. In support of this hypothesis, Forbes et al. [33] showed that the protection mediated by opening of the mitochondrial \( K\text{ATP} \) channel could be blocked by co-administration of the anti-oxidant NAC and Obata and Yamanaka [39] demonstrated that cromakalim or nicorandil increased hydroxyl radical production in rat myocardium. However in our hands diazoxide failed to increase ROS production (Fig. 4) despite being cardioprotective [40]. While this may be due to experimental differences or the sensitivity of our assay it may also indicate that diazoxide acts independently of an increase in free radicals. Nevertheless it is clear that the protection afforded by FCCP acts via generation of an oxidant stress, which could lead to activation of down-stream pathways. The mechanisms that link FCCP treatment and ROS production remain unclear although it is known that interventions that affect electron flow through the electron transport chain have the potential to alter ROS production [38,41–45]. The current understanding of the effects of uncoupling on the electron transport chain suggests that uncoupling agents will decrease mitochondrial ROS production [41,43,46]. This therefore raises the possibility that FCCP-induced free radical production may be from a source other than the mitochondria such as the NADPH oxidase. Separate studies from our laboratory have demonstrated that NADPH oxidase may play a pivotal role in ischaemic preconditioning [47,48] and that thiol modification caused by preconditioning-agonists was blocked with diphenyleneiodo- nium (DPI), which would inhibit NADPH oxidase activity [49]. In addition it has been shown, in mammalian astrocytes, that ROS generation is increased in response to FCCP if applied after activation of NADPH oxidase [50]. It is possible that NADPH oxidase and mitochondria are either independent or interacting sources of ROS although further experiments would be required to clarify this.

The data presented here are the first to show that low concentrations of FCCP can induce anti-ischaemic effects. We have shown that this protection is dependent on ROS and independent of either a fall in bulk cytoplasmic ATP or the activation of \( K\text{ATP} \) channels. This provides further evidence that ROS play an integral role in cardio-protection and the mechanism of their production and downstream targets need further study. Here we provide evidence that mitochondrial uncoupling, as has been suggested to occur in preconditioning, can confer anti-ischaemic effects, although this may only occur at low levels of mitochondrial uncoupling. The effects of FCCP on mitochondrial function are investigated further in the accompanying paper [34].

References