FCCP is cardioprotective at concentrations that cause mitochondrial oxidation without detectable depolarisation

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Abstract

Objective: The role of mitochondria and in particular of mitochondrial uncoupling in the mechanism of cardioprotection is not defined. In the accompanying paper we have shown that pretreatment of isolated rat hearts with a low concentration (100 nM) of FCCP, prior to global ischaemia, is cardioprotective, while 300 nM FCCP exacerbates injury. Here we define the mitochondrial responses to increasing concentrations of FCCP and also to explore the equivalence of the cardioprotective doses of diazoxide.

Methods: Changes in mitochondrial respiration in response to FCCP and diazoxide were determined in isolated rat ventricular myocytes. In addition, mitochondrial state was monitored using confocal microscopy to record mitochondrial potential (TMRM) and redox state (NADH) during FCCP and diazoxide treatment. Myocytes were also voltage-clamped and whole cell currents recorded in response to 100 nM FCCP.

Results: FCCP (10-1000 nM) caused significant dose-dependent increase in oxygen consumption. Diazoxide (30 μM) failed to cause any measurable change in mitochondrial function. FCCP at 100 nM caused mitochondrial oxidation, but no change in mitochondrial membrane potential or &kappa;ATP channel current, while at 300 nM, FCCP caused significant mitochondrial depolarisation. Diazoxide failed to induce any mitochondrial oxidation or depolarisation.

Conclusions: Concentrations of FCCP that cause mitochondrial oxidation without depolarisation are cardioprotective. Higher FCCP concentrations dissipate mitochondrial membrane potential and exacerbate injury. This establishes the principle that mild mitochondrial uncoupling activates a protective mechanism. Diazoxide did not cause mitochondrial oxidation or mitochondrial depolarisation, suggesting it induces protection via another mechanism.

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1. Introduction

It is now clear that mitochondria are not only the site of oxidative phosphorylation and energy production but also play key roles in cell survival and death. Evidence now suggests that mitochondria play a key role in the signalling pathway underlying the cardioprotection mediated by ischaemic preconditioning (reviewed [1]). The observation that protection is blocked by supposedly selective blockers of the mitochondrial ATP-sensitive potassium channel (mitoKÅTP) and mimicked by selective openers implicated this specific channel in the mechanism of protection [2–4]. Several hypotheses have been suggested as to how opening KÅTP channels in the inner mitochondrial membrane may affect mitochondrial function, and how such channel opening could confer protection. It has been suggested that mitoKÅTP channel opening results in mitochondrial depolarisation and this some how confers protection [5]. In line with this it has been shown that hearts can be protected during an ischaemic insult with a pre-treatment of a
mitochondrial uncoupler such as DNP [6,7] and that ischaemic and pharmacological preconditioning has been shown to associate with mitochondrial uncoupling [8]. In the preceding paper we have shown that pretreatment with low concentration FCCP (100 nM) for 5 min prior to a prolonged ischaemic insult significantly improved functional recovery in Langendorff perfused rat hearts [9]. We also showed, however, that a higher concentration (300 nM) not only failed to protect but exacerbated ischaemic injury. It is clear, therefore, that there is a small concentration window in which mild mitochondrial uncoupling is able to induce significant cardioprotection.

Uncoupling of the mitochondria involves the dissociation of electron transport from ATP synthesis; uncoupled respiration continues to generate a proton (H+) efflux, but this is immediately dissipated due to high H+ (or cation) conductance through the membrane, and, without a H+ gradient, ATP synthesis through the F1/F0-ATP synthase is abolished. The hypothesis that mitochondrial uncoupling is a major factor in cardioprotection was first suggested in relation to opening of KATP channels in preconditioning [5,10]. As discussed in our accompanying paper [9] Holmuhamedov et al. have shown in isolated mitochondria and isolated myocytes that KATP channel opening agents induce mitochondrial uncoupling, oxidation and depolarisation, in addition to limiting Ca2+ loading and decreasing the rate of ATP synthesis [5,10]. This is supported by work from separate laboratories which demonstrates that ischaemic preconditioning itself increases the mitochondrial depolarisation seen during the periods of preconditioning and index ischaemia [11] and by previous work showing that a K+ channel opener induces membrane depolarisation of rat liver mitochondria [12] and of mitochondria in permeabilized myocytes [13].

Garlid and colleagues remain the chief opponents to this hypothesis. His group have reported that any uncoupling effects of diazoxide and KATP channel openers are independent of K+ movement, as the openers cause the same effects in Li+ medium, which will not permeate the channel. They also propose that the quantity of K+ moved by the minKATP channel would only be sufficient to cause 1–2 mV depolarisation and that the primary effect of opening minKATP channels is in mitochondrial volume regulation, and related metabolic effects [14]. They also suggest that the effects on mitochondria often reported with diazoxide result from the use of toxic concentrations of the drug, as it has been shown that high concentrations of diazoxide (150 μM) can directly inhibit respiration in the rat heart due to inhibition of succinate dehydrogenase [15], confirmed recently by Hanley et al. [16].

While hearts can be protected during an ischaemic insult by a pretreatment with a mitochondrial uncoupler (FCCP), it has not yet been demonstrated whether the cardioprotective concentration of FCCP used induces any mitochondrial uncoupling, oxidation or membrane depolarisation. Marban’s group have presented data showing diazoxide can induce mitochondrial oxidation in isolated myocytes kept in culture medium for up to 2 d, as assessed by an increase in flavoprotein fluorescence [3]. They interpreted this increase in mitochondrial oxidation as evidence that KATP channels opened in the mitochondria causing mitochondrial uncoupling. Mitochondrial oxidation would occur as a result of uncoupling as electron transport accelerates and continues unrestricted in a futile attempt to maintain ATP. NADH is oxidised to NAD+ and FADH2 oxidised to FAD as part of the process of transferring electrons along the chain, the accelerated state thus causes an oxidised environment within the mitochondria if uncompensated by increased production of electron donors, such as NADH. Thus, there are contradictory hypotheses regarding the effects of opening KATP channels in the inner mitochondrial membrane and how ischaemic preconditioning exerts its cardioprotective effects. In this study, therefore, our aim was to determine the effects on mitochondrial function of FCCP at concentrations that are cardioprotective and to identify the differences between low protective concentrations and higher toxic concentrations, as described in the accompanying paper [9].

FCCP is universally described as a potent mitochondrial uncoupler and, in our accompanying paper [9], we assumed that the low concentrations of FCCP, which confer cardioprotection, were acting to uncouple mitochondrial electron transport from oxidative phosphorylation in a dose-dependent manner. If only a small degree of uncoupling is protective a similar dose response for mitochondrial oxidation and mitochondrial membrane potential might be predicted. It is unclear, however, if there is a linear relationship between uncoupling, oxidation and membrane potential.

Much of the characterisation of FCCP, regarding its effect on mitochondrial membrane potential, has used relatively high concentrations in excess of 1 μM, showing significant mitochondrial depolarisation and complete mitochondrial uncoupling. Seren et al. showed that 17 μM FCCP was necessary to completely collapse the membrane potential in sub-mitochondrial particles prepared from bovine heart [17]. There is only a limited literature regarding lower doses of FCCP as used here [18–20]. The primary aim of the studies described here is to determine the relationships between mitochondrial uncoupling, membrane potential and oxidation and if cardioprotective concentrations of FCCP [9] cause significant effects on redox state or membrane potential in freshly isolated rat ventricular myocytes. An additional objective of these studies was to determine whether diazoxide treatment at cardioprotective concentrations cause changes in mitochondrial oxidation state and membrane potential.

2. Methods and materials

2.1. Myocyte isolation

Ventricular myocytes were isolated from hearts of male Wistar rats (250–300 g) using a standard collagenase digestion protocol as described previously [21]. Isolations where excluded if the fraction of rod shaped myocytes was below 70%. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).
2.2. O₂ consumption measurements

A Clark Oxygen Electrode (Rank Brothers Ltd, UK) was used to measure O₂ consumption as assessed by the change in O₂ concentration of solutions bathing isolated myocytes.

Before each protocol the O₂ electrode was calibrated with saturated (100%) and zero O₂ (plus sodium hydrosulphite (Sigma Chemical Co, UK) solutions. Following isolation, rat ventricular myocytes were stored in the isolation buffer (in mM: NaCl 130, KCl 5.4, NaH₂PO₄ 0.4, HEPES 4.2, Glucose 10, Taurine 20, Creatine 10, CaCl₂ 1.0). 3 ml of cells (approx. 4–6×10⁶ cells) were placed in the incubation chamber and allowed to warm and equilibrate for 15 min. A small magnetic stirrer ensured constant mixing of the myocytes and solution. After 15 min stabilisation, when the rate of O₂ consumption was steady, and linear, the test solution was injected into the chamber, using a glass Hamilton syringe, the pO₂ of the bathing solution was recorded throughout. The temperature within the incubation chamber was maintained at 35±0.1 °C throughout.

Test solutions were FCCP (10 nM–1 μM) and diazoxide (30 μM). In control, a volume of extracellular solution that corresponded with the largest volume of test solution was injected, and controlled for any effect of dilution of the cells on O₂ consumption.

The density and viability of cells in each test group was similar and the rate of O₂ consumption during the stabilisation period, which directly correlates with the number of healthy respiring cells in each sample, was used to normalise the rate of O₂ consumption after treatment.

2.3. Flavoprotein auto-fluorescence recording

Flavoprotein fluorescence was monitored during the treatment period, as a measure of mitochondrial redox state [3,16,22]. Endogenous flavoprotein fluorescence was measured in single isolated rat ventricular myocytes on an inverted Nikon microscope, fluorescence was excited with a xenon arc lamp at 480–505 nm, every 10 s to minimise photobleaching, and the emission at 500–580 nm was recorded by a photomultiplier tube and PowerLab recorder. Cells were allowed to settle in an home-made perfusion bath, and then superfused with a Tyrode solution containing (in mM): NaCl 137, KCl 5.4, MgCl₂ 0.5, HEPES 10, CaCl₂ 1.8, glucose 10, pH 7.4 at 35 °C for a 5 min stabilisation period. The superfusate was switched to test-solution (Tyrode containing either FCCP 10, 30, 100, 300 nM or Control) for 10 min. Cells were then washed with normal Tyrode and a calibration was performed using dinitrophenol (DNP) (100 μM) and sodium cyanide (CN) (4 mM) for 2.5 min each [3]. The change in fluorescence in DNP and CN were respectively defined as 100% and 0% oxidation (complete reduction).

2.4. Whole-cell voltage-clamping

Rat ventricular cardiac myocytes were voltage-clamped and whole-cell current recorded at 35 °C using the perforated-patch technique. The rationale for the use of this technique was to allow the cell to generate its own ATP (rather than to dialyse exogenous ATP into the cell via a ruptured patch). In this way any FCCP-induced deficit in mitochondrial ATP production (and opening of sarcolemmal Kₐ₅₆ channels) could be detected. Electrode resistance was 1–2 MΩ when filled with the standard pipette solution. Pipette solution contained (in mmol/L) KCl₃O₄S 125, NaCl 15, KCl 13, MgCl₂ 1, HEPES 10, pH 7.2 at 35 °C with KOH. Amphoterin B (225 μg/ml) (from Streptomyces, Sigma, UK) in DMSO (0.74% v/v) was added to the pipette solution on the day of use. Extracellular solution was standard Tyrode solution and contained (in mmol/L) NaCl 137, KCl 5.4, MgCl₂ 0.5, HEPES 10, glucose 10, HEPES 10, pH 7.4 at 35 °C.

Following gigaohm seal formation, series resistance was monitored with a repetitive ±5 mV pulse (from ±80 mV holding potential). During membrane permeabilization, series resistance typically fell to 10–15 MΩ within 10 min. Membrane capacitance was recorded at the beginning and end of each experiment. Current was continuously recorded at a sampling frequency of 500 Hz. The control protocol held the voltage at −80 mV and stepped to the test potential of 0 mV for two seconds and back to the holding potential for a further 3 s. This protocol was repeated 5 times. An I–V relationship was then constructed during the repolarising phase of a voltage ramp from +40 to −120 mV (ramp speed 80 mV/s). FCCP (100 nmol/L), or pinacidil (100 μmol/L), was then added and whole cell current recorded and an I–V relationship constructed after 5 min (as above).

2.5. Confocal recording of NADH and FAD auto-fluorescence

Confocal microscopy was utilised to confirm that the flavoprotein fluorescence results obtained using the light microscope were reproducible on the confocal microscope and to determine whether NADH and flavoprotein fluorescence correlated to each other both temporally and in magnitude. Using a Zeiss UV-visible 510 CLSM, NADH autofluorescence and flavoprotein fluorescence were both recorded from isolated rat ventricular myocytes using a protocol that switches the optics rapidly between the two configurations required. Freshly isolated myocytes were allowed to settle for 30 min in a homemade perfusion bath, in standard Tyrode’s solution at room temperature (22 °C). The bath was mounted on the confocal microscope stage and endogenous flavoprotein fluorescence and NADH fluorescence were measured throughout the protocol. Flavoprotein fluorescence was excited at a wavelength of 458 nm and emission recorded at a wavelength>505 nm. NADH autofluorescence was excited at 350 nm and emission between 435 and 485 nm. Myocytes were left for 1 min at which point a steady trace of oxidation and fluorescence had been achieved. FCCP (100 nM) was applied locally to the cell using pressure ejection from a micro-pipette for a period of 5 min or until the signals had reached a steady state. All data were then normalised to the original signal giving a percent change in signal.
from a baseline of 100%. Collected data was analysed using Acquisition Manager (AQM) by Kinetic Imaging.

2.6. Confocal recording of NADH fluorescence and mitochondrial membrane potential ($\Delta\psi$)

Using similar methods to those described above NADH and mitochondrial membrane potential, assessed using TMRM, were measured simultaneously in response to FCCP (100 and 300 nM) or to diazoxide (30 $\mu$M). TMRM is a lipophilic potentiometric dye which partitions between the mitochondria and cytosol in proportion to $\Delta\psi$ by virtue of its positive charge. At low concentrations, the fluorescence intensity is a simple function of dye concentration, which is in turn a direct function of mitochondrial potential. Therefore the accumulation of dye into mitochondria and the intensity of signal is a direct function of mitochondrial potential. Mitochondrial depolarisation then causes the redistribution of dye from mitochondria into the cytosol, causing little change in signal intensity. This redistribution was therefore quantified by measuring the standard deviation of the fluorescence signal, as the concentration of dye into mitochondria causes a bright punctate signal with a high standard deviation of signal across the cell (i.e., very bright pixels over mitochondria, very dark pixels over cytosol) while depolarisation causes a dramatic decrease in the standard deviation of the signal as the dye is evenly distributed throughout the cell (for further discussion and some mathematical modelling, see [26]). Therefore the ratio of mean/SD gives a measure of signal which is dependent on dye localisation to mitochondria and is therefore a highly sensitive measure of subtle changes in mitochondrial potential.

Ventricular myocytes were loaded with TMRM (50 nM for 30 min at room temperature). TMRM-loaded cells were then allowed to settle in a perfusion bath mounted on the stage of the confocal microscope and subsequently perfused with a standard Tyrode’s-type solution, containing TMRM (50 nM), at room temperature (~22 °C). NADH fluorescence and TMRM fluorescence was measured every 10 s throughout the protocol. TMRM was excited using a helium neon laser at a wavelength of 543 nm and emission recorded at wavelengths >560 nm. NADH autofluorescence was excited at 435 and 485 nm. As before, cells were left until steady baseline traces were achieved and the treatment, either FCCP (100 or 300 nM) or diazoxide (30 $\mu$M), each containing TMRM to avoid washout of the dye from the cell were applied locally to the cell using a micropipette for a few minutes until a steady trace was achieved. All experiments were done in the presence of the relevant concentration of TMRM. As before, collected data was analysed using AQM software.

2.7. Materials

Unless otherwise stated all chemicals and FCCP were obtained from Sigma Chemical Co. Ltd. UK. FCCP stocks were made up in DMSO (0.5–150 mM) and then serially diluted to give final concentrations of 10–1000 nM. DMSO was kept...
constant throughout (0.0002%). Diazoxide was obtained in aqueous saline solution (Goldshield Pharmaceuticals Ltd, Croydon, UK) and used at a final concentration of 30 μM.

2.8. Data analysis

Data are generally presented as means ± SEM unless stated otherwise. Data analysis used one way ANOVA followed by a post-hoc Dunnnett’s test to compare treatments to control, or post-hoc Student–Newman–Keuls test to compare between multiple groups. 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. Effect of FCCP and diazoxide on cellular oxygen consumption

O₂ consumption during treatment with either FCCP or diazoxide is shown in Fig. 1. The inset panel shows the change of pO₂ in representative samples was very similar prior to treatment. In all cases the pO₂ at the start of the experiment was 18–21%, there was an initial rise in pO₂, but this was because the electrode is temperature sensitive and the signal increased as the sample warmed to 35 °C. Beyond this warming artefact the oxygen consumption was linear and comparable in all samples prior to treatment. Upon addition of FCCP there was a clear, immediate and dose-dependent increase in oxygen consumption (1 μM FCCP addition to a solution containing no cells did not cause any deflection in O₂ consumption, data not shown). The gradient of the pO₂ trace, which represents the rate of oxygen consumption, before and after treatment was used in the further data analysis. The collated data show the change in the logarithm of the O₂ consumption in response to treatment relative to baseline values for each group.

There is a clear dose-dependent, log-normally distributed, increase in O₂ consumption in response to FCCP treatment at all concentrations tested, indicating significant dose-dependent uncoupling of mitochondrial respiration. The cardioprotective dose of FCCP (100 nM) (see previous paper) caused a 6±0.7 fold increase in O₂ consumption relative to baseline, compared to a marginal decrease in O₂ consumption in control conditions. Diazoxide, on the other hand, did not cause any significant increase in O₂ consumption and hence mitochondrial uncoupling compared to control, at the concentration tested (30 μM).

3.2. Effect of FCCP on flavoprotein auto-fluorescence

The relationship between FCCP treatment and mitochondrial redox state was initially examined in experiments using isolated ventricular myocytes and flavoprotein fluorescence.

![Fig. 3. A: I–V relationships constructed in control conditions and after 5 min exposure to FCCP (100 nM) or pinacidil (100 μM). Lines show representative ramp records and the symbols show means (n=4)± SEM (error bars are largely obscured by the points). The I–V relationship observed under control conditions and following FCCP demonstrate clear inward rectification typical of \( I_{K1} \). Pinacidil substantially linearises the I–V relationship. B: Individual current profiles induced by stepping from −80 to 0 mV for 2 s. C: Whole-cell current (average of the current observed during the final 200 ms of the voltage steps shown in Panel B) normalised to cell capacitance. 100 nM FCCP had no effect on whole-cell current amplitude compared to control (n=4 cells from 3 animals). 100 μM pinacidil induced a large activation (∼7 pA/pF) in whole-cell current in line with activation of the sar\( k_{ATP} \) channel (n=4 cells from 3 animals). D: Chart recording showing the flavoprotein fluorescence in a voltage-clamped rat cardiomyocyte. To prevent bleaching of the flavoprotein auto-fluorescence, recordings were made over a 0.8 s period at 10 s intervals. 100 nM FCCP increased flavoprotein fluorescence rapidly within two minutes while even after 5 min there were no changes in \( k_{ATP} \) channel current (Panels A and C).](image-url)
Fig. 4. Mitochondrial oxidation and membrane potential following FCCP treatment. A: NADH autofluorescence during 5 min exposure to FCCP (100 nM). B: TMRM localisation during 5 min exposure to FCCP (100 nM). 100 nM FCCP caused significant mitochondrial oxidation without causing any redistribution in TMRM. C: NADH autofluorescence during 5 min exposure to FCCP (300 nM). D: TMRM localisation during 5 min exposure to FCCP (300 nM). 300 nM FCCP caused both mitochondrial oxidation and mitochondrial depolarisation as shown by decreased NADH fluorescence and decreased SD of the TMRM signal. In all cases FCCP was applied at 0 s following a baseline recording. E: Average data of NADH autofluorescence following FCCP treatment. Both 100 nM and 300 nM cause significant reduction in NADH signal indicating mitochondrial oxidation. Data are mean + SEM. n=9 per group. *p<0.05 cf. baseline. F: Change in standard deviation of TMRM distribution in response to FCCP treatment. 100 nM caused no change in SD of TMRM whereas 300 nM caused a significant decrease in the SD of TMRM across the cell indicating mitochondrial depolarisation. Data are mean + SEM. n=8 per group (100 nM) and n=9 per group (300 nM). *p<0.05 cf. Baseline.
3.3. **Effect of FCCP on sarcolemmal KATP channel currents**

In separate experiments whole-cell currents were measured during FCCP (100 nM) treatment using the perforated patch technique. Since $K_{\mathrm{ATP}}$ channel opening has previously been suggested to be cardioprotective, it could be argued that an FCCP-induced depletion of cellular ATP could open sarcolemmal $K_{\mathrm{ATP}}$ channels and hence, indirectly, induce protection. Using the perforated patch technique, the cell remains essentially undialysed and intracellular ATP is maintained by provision of exogenous glucose. **Fig. 3A** shows $I-V$ relationships constructed under control conditions and following 5 min FCCP (100 nM). Under these conditions the $I-V$ relationship demonstrates clear inward rectification typical of $K_{\mathrm{ATP}}$, which is substantially linearised when $K_{\mathrm{ATP}}$ channels are opened by pinacidil. 5 min exposure to FCCP (100 nM) was, however, without effect. Individual current traces are shown in **Fig. 3B** showing the activation of $K_{\mathrm{ATP}}$ channel current at 0 mV by pinacidil. FCCP (100 nM) had no effect on whole-cell current amplitude compared to control ($n=4$), whereas 100 μM pinacidil induced a large activation (~7 pA/pF) in whole-cell current in line with activation of the sarcolemmal $K_{\mathrm{ATP}}$ channel ($n=4$). **Fig. 3D** shows flavoprotein fluorescence measured in separate experiments in voltage-clamped myocytes during an equivalent 5 min exposure for FCCP (100 nM) showing that over this time scale changes in flavoprotein fluorescence (Panel D) are not associated with $K_{\mathrm{ATP}}$ channel opening (Panels A and C).

3.4. **Simultaneous measurement of NADH auto-fluorescence and mitochondrial membrane potential**

To determine the relationship between mitochondrial redox state and mitochondrial membrane potential, NADH auto-fluorescence and TMRM fluorescence were recorded simultaneously during FCCP and diazoxide treatment. Prior to this, in separate experiments, flavoprotein and NADH auto-fluorescence were measured simultaneously following FCCP (100 nM) treatment using confocal microscopy. Oxidation of NADH caused a reduction in fluorescence intensity while oxidation of FADH increased fluorescence intensity with an identical time course (data not shown). NADH fluorescence was chosen over flavoprotein fluorescence as the indicator of mitochondrial redox as there is less overlap between the emission spectra of NADH (peak ~450 nm) and of TMRM (peak at ~590 nm) compared to that of flavoproteins (peak emission ~560 nm).

**Fig. 4** shows confocal images, of both NADH auto-fluorescence (Panel A) and the SD of TMRM signal (Panel B), from a myocyte before and after treatment with the cardioprotective concentration of 100 nM FCCP as well as the continuous record of fluorescence during exposure. The arrows indicate the time-point on the relevant trace that these images were taken. 100 nM FCCP caused mitochondrial oxidation with no detectable depolarisation. Panels C and D show confocal images, of NADH autofluorescence and SD of TMRM signal, from a myocyte before and after treatment with the higher, cardiotoxic (see accompanying paper) [9], concentration of 300 nM FCCP again arrows indicate the time-point on the relevant trace that these images were taken. In contrast to the effect of 100 nM FCCP, 300 nM caused both mitochondrial oxidation and mitochondrial membrane depolarisation. Panel E shows the mean data (9 myocytes from 3 separate cell isolations) for NADH autofluorescence changes in response to 100 and 300 nM FCCP. In these confocal experiments, FCCP oxidised NADH reducing baseline fluorescence to 44±4% and 35±6% of baseline in 100 and 300 nM FCCP respectively. Panel F, shows the mean data for the relationship between FCCP concentration and mitochondrial membrane potential, measured as TMRM SD. There was no detectable redistribution of TMRM in response to 100 nM FCCP. In contrast, 300 nM FCCP caused significant reduction in the SD of TMRM signal, indicating a redistribution of the dye from the mitochondria into the cytosol in response to mitochondrial membrane depolarisation. Similar results were also found using the dequench method [23] of TMRM analysis (using 3 μM TMRM, data not shown). Thus, mitochondrial redox state does not directly correlate with mitochondrial membrane potential and large changes in mitochondrial oxidation can occur without significant changes in mitochondrial membrane potential.

**Fig. 5**, shows the NADH and TMRM fluorescence data from myocytes treated with 30 μM diazoxide. Diazoxide did not cause any mitochondrial oxidation or mitochondrial depolarisation at the concentration tested.

4. **Discussion**

This study demonstrates that low concentrations of FCCP are effective at uncoupling mitochondria in a dose–dependent manner. FCCP as low as 10 nM, caused a detectable increase in cellular O$_2$ consumption, and therefore a detectable level of uncoupling. In line with the hypothesis that low concentrations of FCCP would only cause mild uncoupling, the cardioprotective concentration of FCCP (100 nM) caused significantly less mitochondrial uncoupling than the higher doses of FCCP (≥300 nM) previously shown to have toxic effects on isolated rat hearts. However, diazoxide, at a concentration that has previously been shown to be cardioprotective [2], did not alter...
O₂ consumption. This is contrary to the initial hypothesis and the implication of other studies that diazoxide induces cardioprotection by partially depolarising the mitochondria. Minners et al. [8] previously demonstrated that the same concentration of diazoxide as used in this study caused a significant increase in O₂ consumption as well as decreasing mitochondrial membrane potential and decreasing cellular levels of ATP, in two different cell lines (Girardi cells and C2C12 myotubes). It is possible that this disparity may reflect differences between the cultured cells used by Minners et al. and the freshly isolated ventricular myocytes used in the present study. However, our data are supported by studies by Grover et al., who have shown that, at pharmacological doses, other K<sub>ATP</sub> openers have no effect on the efficiency of oxygen utilisation in the intact heart, which would exclude mitochondrial uncoupling as a mechanism of action [24,25].

It therefore seems likely that diazoxide, and FCCP-induced mild uncoupling, operate to induce protection through distinct mechanisms, at least at the level of the mitochondria. However, it is possible that downstream effectors or kinase pathways may be common to both mechanisms. Further to this, the cardioprotective concentration of FCCP did not activate sarcolemmal K<sub>ATP</sub> channels within the time-course of the pretreatment period. Whilst we are unable to say from this alone whether mitochondrial K<sub>ATP</sub> channels also did not open, this combined with the observations in the accompanying paper (that protection is not blocked by K<sub>ATP</sub> channel blockers and bulk ATP did not fall during FCCP treatment) suggests that FCCP exerts its cardioprotective effects independent of K<sub>ATP</sub> channel activation.

Cardioprotective and toxic doses of FCCP both caused mitochondrial oxidation, however, 100 nM FCCP (which was cardioprotective) had no measurable effect on mitochondrial membrane potential while 300 nM FCCP (a cardiotoxic concentration) caused mitochondrial depolarisation. Not only did 100 nM cause mitochondrial oxidation without depolarisation but the depolarisation that occurred in the 300 nM treated cells occurred significantly after mitochondrial oxidation. This suggests that in response to low concentrations of FCCP, where the proton shunt may be relatively modest, stimulation of respiration may increase proton pumping by the respiratory chain sufficiently to maintain the potential, while causing maximal oxidation of NADH and flavoproteins. The study, therefore, provides evidence that the relationship between mitochondrial oxidation and mitochondrial membrane potential is not linear and it is possible to cause oxidation without detectable depolarisation. Clearly we are unable to discount changes in membrane potential below the detection limits of our experimental set up. However, mathematical models of TMRM behaviour [26] suggest that the resolution of detection for altered potential is around 5 mV thus it seems unlikely that changes below this threshold could have mechanistic significance.

Cardioprotection, is only seen at an FCCP concentration (100 nM) that oxidises but does not depolarise the mitochondria. This indicates that protection induced by mitochondrial uncoupling has a small concentration window, in which mild uncoupling of the mitochondria activates an intrinsic protection mechanism but more severe uncoupling results in mitochondrial depolarisation and cell death. In addition, diazoxide, at a concentration previously shown to be cardioprotective in the isolated rat heart, and still within the accepted pharmacological range, caused no mitochondrial oxidation nor any detectable mitochondrial depolarisation. These are results which reflect historical findings within our laboratory as we have been unable to detect any changes in flavoprotein fluorescence in response to diazoxide (data not shown). This also agrees with O₂ consumption data in which diazoxide failed to show the uncoupling effect that might have been expected to follow an increase in the K⁺ leak in to the mitochondria. It is possible that the flux of K⁺, which enters the mitochondria in response to mitoK<sub>ATP</sub> opening with diazoxide, is not sufficient to cause either mitochondrial oxidation or depolarisation. These are results which reflect historical findings within our laboratory as we have been unable to detect any changes in flavoprotein fluorescence in response to diazoxide (data not shown). This also agrees with O₂ consumption data in which diazoxide failed to show the uncoupling effect that might have been expected to follow an increase in the K⁺ leak in to the mitochondria. It is possible that the flux of K⁺, which enters the mitochondria in response to mitoK<sub>ATP</sub> opening with diazoxide, is not sufficient to cause either mitochondrial oxidation or depolarisation. These are results which reflect historical findings within our laboratory as we have been unable to detect any changes in flavoprotein fluorescence in response to diazoxide (data not shown).
protective pathways. Our results suggest that neither FCCP nor diazoxide act via the mechanism that requires substantial mitochondrial depolarisation as suggested by Holmuhamedov et al. [10].

In conclusion, the studies presented here provide evidence that the relationship between mitochondrial oxidation and mitochondrial membrane potential is complex, and that at low concentrations, an increased respiratory rate compensates for the proton leak, causing oxidation without depolarisation. Treatment of cells with low concentrations of FCCP sufficient to cause mitochondrial oxidation without any detectable change in mitochondrial membrane potential, protects the heart against ischaemic injury while higher concentrations dissipate mitochondrial membrane potential and exacerbate injury. This indicates that protection induced by mitochondrial uncoupling has a small window, in which mild uncoupling of the mitochondria activates an intrinsic protection mechanism but more severe uncoupling results in mitochondrial depolarisation and cell death. However, it has also been shown that a cardioprotective concentration of diazoxide did not cause either mitochondrial oxidation or mitochondrial depolarisation. The protection afforded by low concentrations of FCCP was also shown in the accompanying paper to occur in the absence of any change in bulk ATP concentration or opening of sarcolemmal K_ATP channels and to involve a free-radical dependent mechanism.

References


