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The role of oxidized cytochrome c in regulating mitochondrial reactive oxygen species production and its perturbation in ischaemia

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Short title: Cytochrome c regulates mitochondrial ROS

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Non-standard Abbreviations and Acronyms
ANT, adenine nucleotide translocase; CsA, cyclosporine A; DEA, diethylamine NONOate diethylammonium salt; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; GMS, 5 mM L-glutamate + 2 mM L-malate + 5 mM succinate; HK, hexokinase; IMS, intermembrane space; IP, ischaemic pre-conditioning; mPTP, mitochondrial permeability transition pore; OMM, outer mitochondrial membrane; SOD, superoxide dismutase.

Key words
cytochrome c, superoxide, mitochondrial permeability transition pore, hexokinase, Bcl-xL
ABSTRACT
Oxidized cytochrome c (Cyt-c) is a powerful superoxide scavenger within the mitochondrial intermembrane space, the importance of this role in situ has not been well explored. Here we investigate this with particular emphasis on whether loss of Cyt-c from mitochondria during heart ischaemia may mediate the increased production of reactive oxygen species during subsequent reperfusion that induces mitochondrial permeability transition pore (mPTP) opening. Mitochondrial Cyt-c depletion was induced in vitro with digitonin, or by 30 minutes ischaemia of the perfused rat heart. Control and Cyt-c-deficient mitochondria were incubated with mixed respiratory substrates and an ADP-regenerating system (State 3.5) to mimic physiological conditions. This contrasts with most published studies performed with a single substrate and without significant ATP turnover. Cyt-c-deficient mitochondria produced more H$_2$O$_2$ than control mitochondria, and exogenous Cyt-c addition reversed this increase. In the presence of increasing [KCN] rates of H$_2$O$_2$ production by both pre-ischaemic and end-ischaemic mitochondria correlated with the oxidized Cyt-c content but not with rates of respiration or NAD(P)H autofluorescence. Cyt-c loss during ischaemia was not mediated by mPTP opening (cyclosporine-A insensitive); nor was it associated with changes in mitochondrial Bax, Bad, Bak or Bid. However, bound hexokinase 2 and Bcl-xl were decreased in end-ischaemic mitochondria. We conclude that Cyt-c loss during ischaemia, caused by outer membrane permeabilisation, is a major determinant of H$_2$O$_2$ production by mitochondria under pathophysiological conditions. We further suggest that in hypoxia production of H$_2$O$_2$ to activate signaling pathways may be also mediated by decreased oxidized Cyt-c and less superoxide scavenging.
INTRODUCTION

Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide are known to play important signaling roles when present at low concentrations, but at higher concentrations, especially under conditions that lead to the formation of hydroxyl radicals, they can cause major damage to cellular components that ultimately cause cell death (see [1]). Superoxide is generated at a variety of sites both within the mitochondrial matrix and in the intermembrane space (IMS) making mitochondria a major source of intracellular ROS (see [1-3]). The majority of the superoxide formed in both compartments is believed to be converted into hydrogen peroxide by the manganese (matrix) and zinc (IMS) superoxide dismutase (SOD). The hydrogen peroxide is then removed by different enzymes (i.e. glutathione peroxidases, catalase and the family of the peroxiredoxins) which use the intramitochondrial pool of NADPH and GSH (see [4] for further detail). In the presence of ferrous ions this pathway of superoxide removal runs the risk of generating the highly damaging hydroxyl radical from hydrogen peroxide through the Fenton Reaction [2]. However, in the IMS there is another potential mechanism for superoxide removal that involves its conversion back to oxygen by oxidized cytochrome c (see [1,3]). Cytochrome c is present at about 1 mM in the IMS and can be reduced by superoxide with a rate constant of about $10^7 \text{ M}^{-1}\text{s}^{-1}$ [3, 5]. The reduced cytochrome c may then be rapidly reoxidized by cytochrome c oxidase (complex IV) to regenerate oxidized cytochrome c that can remove more superoxide. Experiments with isolated mitochondria [6-7] and complex IV reconstituted into proteoliposomes [8] have confirmed that cytochrome c can act as an efficient superoxide scavenging system. However, the physiological importance of cytochrome c for redox scavenging has not been rigorously tested.

In this paper we report the results of such studies performed in the context of the mitochondrial dysfunction that occurs following ischaemia and reperfusion of the heart which is associated with oxidative stress caused by mitochondrial overproduction of ROS [9-11]. Oxidative stress, together with calcium-overload, induce opening of the mitochondrial Permeability Transition Pore (mPTP) which is known to be a critical event in reperfusion injury and causes the necrotic cell death characteristic of myocardial infarction [12]. Although the detrimental effects of prolonged ischaemia on the respiratory chain are well established, the mechanisms responsible for increased ROS production are less clear [2, 13-14]. However, loss of cytochrome c from the mitochondria has been reported to occur during ischaemia [13, 15], and this, together with a decrease in its oxidation state, could provide the mechanism. In this paper we explore this possibility more rigorously. The Qo site of complex III can be a major site of superoxide production into the intermembrane space where cytochrome c resides [2]. Thus cytochrome c loss would lead to less superoxide scavenging by this route leading to greater production of hydrogen peroxide through SOD and hence the possibility of greater oxidative stress. Furthermore, to maintain electron flow into cytochrome oxidase, the remaining cytochrome c in the IMS would become more reduced leaving even less oxidized cytochrome c to scavenge superoxide. In addition, as cytochrome c reduction state increase so would that of complex 1, potentially increasing matrix production of superoxide, $\text{H}_2\text{O}_2$ and hydroxyl radicals [2]. Here we demonstrate directly that when mitochondria are incubated under conditions that support rates of oxidative phosphorylation similar to those in situ, both the amount and the reduction state of cytochrome c do influence mitochondrial $\text{H}_2\text{O}_2$ production and that loss of cytochrome c from mitochondria during ischaemia can explain the observed increase in $\text{H}_2\text{O}_2$ production. This cytochrome c loss occurs without mPTP opening but is associated with a loss of bound hexokinase 2 (HK2) and a depletion in Bcl-xl content that are implicated in the regulation of OMM permeability that leads to cytochrome c release in apoptosis [16-17].

MATERIALS AND METHODS

Antibodies and chemicals

The following antibodies were used in this study: Bax B-9 (mouse monoclonal, Santa Cruz), Bak (rabbit monoclonal, Abcam), Bad and P-Bad (rabbit monoclonal, Cell Signaling), Bid (goat polyclonal, R&D Systems), t-Bid (rat monoclonal, R&D Systems), hexokinase 1 (mouse monoclonal, Chemicon Milipore), hexokinase 2 (rabbit monoclonal, Cell Signaling),
cytochrome c (mouse monoclonal, BD Biosciences Pharmingen), Bcl-xL (rabbit monoclonal, Cell Signaling, #2764). All chemicals used in this study were purchased from Sigma unless otherwise stated.

**Heart Perfusion.**
All procedures conformed to the UK Animals (Scientific Procedures) Act 1986. Male Wistar rats (225-250 g) were killed by stunning and cervical dislocation and hearts (~0.75 g) were rapidly removed into ice-cold Krebs-Henseleit buffer containing (mmol / L) NaCl 118, NaHCO$_3$ 25, KCl 4.8, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, glucose 11 and CaCl$_2$ 1.2 gassed with 95% O$_2$ / 5% CO$_2$ at 37°C (pH 7.4). Langendorff heart perfusions were performed as described previously [18]. All hearts were subject to 30 min pre-ischaemia, which included 10 minutes treatment with 0.2 µM CsA if required, as shown schematically in Figure 1. Global normothermic ischaemia (index ischaemia) was induced by halting perfusion for 30 min and immersing the heart in perfusion buffer at 37°C. At the end of the pre-ischaemic or ischaemic period the hearts were either removed from the perfusion cannula for the preparation of mitochondria or saponin-permeabilized fibres, or freeze-clamped using liquid-nitrogen cooled tongues. In the latter case, the hearts were ground under liquid nitrogen, and stored at -80°C for later analysis.

**Isolation of mitochondria.**
Two different protocols were employed for mitochondrial preparation involving either polytron tissue homogenization or protease treatment followed by Dounce Potter homogenization. The latter gave more mitochondria with less loss of cytochrome c and was used for functional assays. However, this technique was not suitable for determining proteins bound to the OMM because of their degradation by the protease treatment. In both cases all steps were performed at 4°C.

**Protease method:** Each heart was rapidly chopped into fine pieces with scissors before incubating at 4°C for 7 min with stirring in 25 ml isolation buffer (ISA: 300mM sucrose, 2mM EGTA, 10mM Tris-HCl, pH 7.1 at 4°C) containing 0.1 mg/ml of bacterial proteinase type XXIV (Sigma). The resulting tissue suspension was poured into a 50 ml glass Potter homogenizer and homogenized for 3 min using a motorized Teflon pestle. The homogenate was centrifuged at 7,500 g for 7 min and the resulting pellet rinsed twice with 5 ml isolation buffer, resuspended in 20 ml isolation buffer and subject to further homogenization as described above. The homogenate was then centrifuged at 700 g for 10 min and the resultant supernatant centrifuged at 7,000 g for 10 min to yield a crude mitochondrial pellet that was resuspended in ISA containing 25% (w/v) Percoll (pH 7.1-7.2 at 4°C) and centrifuged at 17,000 g for 10 min. The resulting pellet was resuspended with ISA and centrifuged again at 7,000 g for 10 min. The final purified mitochondrial pellet was resuspended in ISA and the protein concentration determined by Biuret method using bovine serum albumin (BSA) as a standard. Mitochondria were kept on ice at a final concentration of 50 mg / ml for no more than 4 hours.

**Polytron method:** This was performed essentially as described previously [19]. Each heart was homogenized at 4°C in 6 ml ISA using a Polytron tissue disruptor (Kinematica) at 10,000 rpm for 2 bursts of 5 s and 1 of 10 s. The homogenate was diluted with 3 volumes of ISAPP (ISA supplemented with inhibitors of proteases (Roche complete) and phosphatases (Sigma cocktail 1) and further homogenized for 2 min in a 50 ml glass Potter homogenizer as above. The resulting homogenate was centrifuged at 7,500 g for 7 min. For the preparation of density-gradient purified mitochondria the resultant pellet was processed exactly as described above, except that the isolation buffer used was supplemented with inhibitors of proteases and phosphatases.

**Preparation of cytosolic fractions.**
Frozen heart powder was suspended at 50 mg per ml isolation buffer containing protease and phosphatase inhibitors and sonicated three times in 5 s bursts followed by centrifugation at 16,000 g for 10 s in a microcentrifuge to remove cell debris. The resulting supernatant, considered as the cytosolic fraction, was dissolved in SDS-PAGE sample buffer and normalized to 4 mg / ml using a Bicinchoninic acid-based (BCA) protein assay (Pierce).
**Permeabilized cardiac fibres preparation.**

Preparation of permeabilized cardiac left ventricular fibres was performed using well established protocols [20-22]. Small pieces of cardiac muscle were taken from the left ventricle to prepare permeabilized fibres at different points of the perfusion protocols as shown by the arrows in Figure 1. All procedures were carried out at 4°C. The samples were rapidly dissected into bundles of fibres and incubated with stirring in 3 ml of solution A (see below) containing saponin (50 µg / ml) before washing twice for 10 min in solution B (see below).

Solution A in mmol / L: CaK₂EGTA 2.77; K₂EGTA 7.23 (pCa = 7); MgCl₂ 6.56; dithiotreitol (DTT) 0.5; MES 50; imidazole 20; taurine 20; Na₂ATP 5.3; creatine phosphate 15. The pH was adjusted to 7.1 at room temperature with 10 M KOH.

Solution B in mmol / L: CaK₂EGTA 2.77; K₂EGTA 7.23 (pCa =7); MgCl₂ 1.38; DTT 0.5; MES 100; imidazole 20; taurine 20; KH₂PO₄ 3. The pH was adjusted to 7.1 at room temperature with 10 M KOH and 2 mg / ml BSA added.

**Digitonin treatment of mitochondria.**

When required, partial permeabilization of the outer membrane to cytochrome c was achieved using digitonin. The quantity of digitonin used was defined by titration in order to obtain the same sensitivity of the respiration to exogenous cytochrome c in control mitochondria as exhibited by mitochondria from hearts subject to 30 min ischaemia. This was found to be 180 µg digitonin per 250 µg mitochondria in 2 ml KCl incubation medium at 37°C.

**Measurements of respiration.**

Oxygen consumption of isolated mitochondria or permeabilized skinned fibres was measured polarographically at 37°C in an Oroboros Oxygraph (Graz, Austria). Unless stated otherwise the respiratory substrate was a mixture of 5 mM L-glutamate, 2 mM L-malate and 5 mM succinate (GMS) to ensure electron entry at both complex I and complex II of the respiratory chain as occurs in vivo. Rates of respiration were determined before and after addition of 1.5 mM ADP (State 2 and State 3 respectively) or at an intermediate rate (State 3.5 - isolated mitochondria only) established by addition of ATP and creatine in the presence of 160 µg creatine kinase per mg mitochondria to mimic ATP turnover in vivo. The permeability of the mitochondrial outer-membrane to cytochrome c was assessed by addition of exogenous cytochrome c (10, 25 or 50 µM as indicated in legend to figures) after ADP or ATP + creatine.

**Measurement of hydrogen peroxide production and NAD(P)H autofluorescence.**

The rate of H₂O₂ production was determined with the fluorescent hydrogen peroxide indicator, Amplex Red (30 µM), in the presence of peroxidase using excitation and emission wavelengths of 540 and 585nm respectively. Measurements were made under State 3.5 conditions (identical to those used for respiration) in a multi-well fluorescence plate reader (Flexstation, Molecular Devices). To each of 8 wells in a 96 well plate were added 200 µl of a mitochondrial solution (0.25 mg protein / ml) in the KCl buffer used for respiration studies but containing 30 µM Amplex Red and 0.1 mg/ml peroxidase. After baseline recording, a 10 µl aliquot of substrate solution (105 mM L-glutamate, 42 mM L-malate, 105 mM succinate, 4.2 mM ATP, and 105 mM creatine, in KCl buffer at pH 7.3) was automatically added. The rate of H₂O₂ production was calibrated using a standard curve generated under the same experimental conditions with additions of 0-40 pmoles exogenous H₂O₂ (see supplementary Figure S6). It was confirmed that addition of exogenous superoxide dismutase (SOD), to ensure conversion of superoxide to H₂O₂, was without effect on rates of H₂O₂ production in State 4 or State 3.5. However at the highest rates observed upon addition of antimycin A, where part of the superoxide is produced in the IMS, a slight increase (7%) was observed (Supplementary Fig S1). Indeed under these conditions exogenous cytochrome c gave a substantial reduction in H₂O₂ production (Supplementary Fig. S2B). Taken together these data suggest that normally rates of superoxide production in the IMS are sufficiently low for complete dismutation to H₂O₂ in the IMS either spontaneously or by SOD. However, when antimycin A is added, rates of superoxide production in the IMS are high enough for some to
escape across the OMM and undergo dismutation to hydrogen peroxide that is accelerated by added SOD. This superoxide can also be removed by exogenous cytochrome c leading to a reduction in the H₂O₂ detected.

The redox state of NAD(P)H was determined using autofluorescence (340/460 nm) under similar conditions. The NAD(P)H signal was normalized to the minimum and maximum values obtained with 1 µM FCCP and 1.5 mM KCN, respectively.

**Spectrophotometric measurement of cytochrome c reduction state.**

The reduction state of cytochrome c was monitored using a custom built double-beam spectrophotometer with a wavelength pair of 550 / 540 nm as described previously [23]. The use of this wavelength pair corrects the increase in absorbance at 550 nm occurring upon cytochrome c reduction for any non-specific light scattering changes that are mirrored in the change in absorbance at 540 nm, the isosbestic point for oxidized and reduced cytochromes in intact mitochondria. Mitochondria (0.285 mg / ml) were incubated at 37°C with constant stirring in 3.5 ml standard KCl buffer under different respiratory conditions and A₅₅₀₋₅₄₀ monitored continuously. Additions were made through an injection port as required concluding with 3.5 mM FeK₃(CN)₆ followed by 7 mM Na₂S₂O₄ to determine the fully oxidized and reduced A₅₅₀/₅₄₀ respectively. The extinction coefficient for reduced minus oxidized cytochrome c at this wavelength pair was taken as 19.1 L:mmol⁻¹·cm⁻¹ [24].

**Measurement of cytochrome c, Bax, Bak, Bad/P-Bad, Bid/t-Bid, Bcl-xl and hexokinase by Western blotting.**

Mitochondria and cytosolic fractions, prepared by the polytron method (see above), were separated by SDS-PAGE (12 % for cytochrome c and Bcl-xl, 10% for Bak and Bax, and 5% for hexokinase) using 20 µg protein for each track (40 µg for Bak). Gels were then subjected to Western blotting with the required primary antibody and blots developed using the required Ig horseradish peroxidase secondary antibody, with ECL/ECL+ detection (Amersham Biosciences UK Limited). Appropriate exposures of the film were used to ensure that band intensities were within the linear range. Quantification of blots was performed using an AlphaInotech ChemiImager 4400 to image the blot, and analysis of band intensity with AlphaEase v5.5 software. Each blot contained samples of control and end-ischaemic mitochondria to allow direct comparisons between groups using the same film exposure. In order to normalize band intensities, parallel blots were performed on the same samples using antibodies against the adenine nucleotide translocase (ANT).

**Measurement of caspase 3 cleavage and enzyme activity.**

Caspase 3 cleavage was studied using a cytosolic fraction prepared from frozen heart powder (see above) and analyzed by Western blotting using a polyclonal antibody that detects full length caspase 3 and the large fragment of caspase 3 resulting from cleavage (Cell Signaling, #9662). Band intensities were normalized using ANT and GAPDH for the mitochondrial and cytosolic samples, respectively. Caspase 3 activity was determined using a kit (CASP3, Sigma-Aldrich) according to the manufacturer’s instructions. This assay detects cleavage of the caspase 3 substrate Ac-DEVD-p-nitroaniline by measuring the absorbance (405 nm) of the p-nitroalanine product. Samples of the cytosolic fraction obtained from the frozen heart powder (see above), were incubated at 0.5 mg protein / ml in the assay buffer at 37°C and the formation of p-nitroalanine measured every 30 min for 3 hours. Caspase 3 activity was also monitored in the presence of 20 µM DEVD-CHO, an inhibitor of caspase 3 that totally abolished the activity of the recombinant caspase 3 provided in the kit. Caspase 3 activity was calibrated by measuring the absorbance of known concentrations of p-nitroalanine.

**Measurement of hexokinase, citrate synthase and complex I specific activity.**

Aliquots (0.75 mg protein) of frozen mitochondria prepared by the polytron method (see above) were solubilized by brief sonication at 4°C and 2 mg / ml in buffer containing 33 mM KH₂PO₄ and 50 µM DTT (pH 7.2). For assay of hexokinase, samples (20, 30 or 40 µl) were added to 1 ml assay buffer (pH 7.4) containing 100 mM Tris-HCl, 0.4 mM NADP⁺, 10 mM MgCl₂, 5 mM ATP, 0.3% (v/v) Triton X-100 and 0.5 units / ml of G6PDH and incubated for 2 min at 37°C before addition of glucose (1 mM final) to start the reaction. Hexokinase activity
was calculated from the rate of NADPH production corrected for glucose-independent rates of NADPH formation determined in parallel assays lacking glucose. Citrate synthase and Complex I activity were determined as described previously [25].

Statistical Analysis:
Data are presented as means ± S.E.M. Statistical significance was evaluated using 1-way ANOVA (Kaleidagraph, 4.03), and differences were considered significant at \( p < 0.05 \).

RESULTS
Loss of cytochrome c increases mitochondrial \( \mathrm{H}_2\mathrm{O}_2 \) production
In order to study the relationship between \( \mathrm{H}_2\mathrm{O}_2 \) production and the content of cytochrome c, we first established experimental conditions under which the rate of oxygen consumption had a minimal impact on this parameter. This is important since it is well known that rates of ROS production in isolated mitochondria are closely linked to both the proton motive force (\( \Delta \mathrm{p} \)) and the ratio NADH to NAD\(^+\) [2]. Consequently, \( \mathrm{H}_2\mathrm{O}_2 \) emission and oxygen consumption from control mitochondria were determined after addition of increasing concentrations of the protonophore FCCP to increase mitochondrial respiration. As illustrated in Figure 2A, the rate of \( \mathrm{H}_2\mathrm{O}_2 \) production was decreased linearly as mitochondrial respiration increased from basal to 270 nmol \( \mathrm{O}_2 \)/min/mg protein, and thereafter showed no significant decrease as respiration was increased from 270 to 380 nmol \( \mathrm{O}_2 \)/min/mg protein. This rate of respiration corresponds to half the maximal rate obtained after addition of a saturating [ADP] (see Figure 3A). In order to mimic physiological rates of ATP turnover, oxygen consumption was increased to 380-400 nmol \( \mathrm{O}_2 \)/min/mg protein by incubating mitochondria with an ADP-regenerating system containing creatine and creatine kinase (Figure 2B). Under such conditions, mitochondrial respiration was controlled by the amount of creatine kinase added (i.e. 160 \( \mu \)g / mg mitochondria), and this intermediate respiratory rate is referred to as State 3.5. Cytochrome c loss was then induced by addition of digitonin to permeabilize the mitochondrial outer membrane without damaging the inner membrane, and rates of mitochondrial \( \mathrm{H}_2\mathrm{O}_2 \) production determined in State 3.5. It should be noted that under these more physiological conditions rates of \( \mathrm{H}_2\mathrm{O}_2 \) production were only about 12% of those in State 4 used in the majority of published studies on the regulation of mitochondrial ROS production (Supplementary Fig. S1). After digitonin addition, mitochondrial respiration was decreased by 14%, and \( \mathrm{H}_2\mathrm{O}_2 \) production increased by 45% when compared to corresponding controls (Figures 1B and C). After addition of 10 \( \mu \)M exogenous cytochrome c both parameters were brought back to control values. As predicted, the impairment of electron flow caused by cytochrome c loss was also associated with an increase of NAD(P)H autofluorescence (Figure 2D). To evaluate the impact of NAD(P)H redox state on \( \mathrm{H}_2\mathrm{O}_2 \) emission in State 3.5, 5 \( \mu \)M potassium cyanide was added to mimic the effects of digitonin on both the rate of oxygen consumption and NAD(P)H autofluorescence (Figure 2B and D). Unlike the effect of digitonin, the increase in NAD(P)H autofluorescence after cyanide addition was not accompanied by any increase in the rate of \( \mathrm{H}_2\mathrm{O}_2 \) production (Figure 2C). We conclude that loss of cytochrome c from mitochondria increases \( \mathrm{H}_2\mathrm{O}_2 \) emission. Note that even in control mitochondria, addition of exogenous cytochrome c was able to decrease \( \mathrm{H}_2\mathrm{O}_2 \) emission slightly without exerting a significant effect on the rate of respiration. This may reflect the leakage of a small amount of superoxide into the medium and its rapid removal by exogenous cytochrome c before spontaneous dismutation to \( \mathrm{H}_2\mathrm{O}_2 \) and detection by Amplex Red.

Mitochondrial loss of cytochrome c during ischaemia occurs without mPTP opening
Hearts were perfused ± 0.2 \( \mu \)M cyclosporine A (CsA) prior to ischaemia and mitochondria isolated either before (Pre) or after ischaemia (Isch; Isch+CsA) as illustrated schematically in Figure 1. End-ischaemic mitochondria exhibited lower rates of respiration induced by saturating [ADP] (State 3) than pre-ischaemic mitochondria (Figure 3A), but this decrease was reversed after addition of 10 \( \mu \)M exogenous cytochrome c. Saponin-permeabilized fibres prepared from ischaemic hearts also showed a stimulation of respiration by added cytochrome c that was not observed in control fibres (Figure 3B). This is important because
it confirms that the cytochrome c loss observed after 30 minutes ischaemia was not an artefact of mitochondrial isolation. Nevertheless, the results shown in Figures 2 and 3 demonstrate that the mitochondrial isolation procedure itself is associated with a slight loss of exogenous cytochrome c but this was no greater in end-ischaemic than pre-ischaemic mitochondria. Thus in pre-ischaemic and end-ischaemic permeabilized fibres respiration was stimulated by exogenous cytochrome c by zero and 25 ± 2% respectively whilst the equivalent stimulation in isolated mitochondria was 8 ± 1% and 32 ± 2% respectively; in both cases the increase caused by ischaemia was about 25%. Cytochrome c leak was not prevented by 10 min of pre-ischaemic perfusion of hearts with 0.2 µM CsA (Figure 3B), a treatment that we have shown previously inhibits mPTP opening both in situ and in subsequently isolated mitochondria [26]. We also confirmed that respiration was still sensitive to cytochrome c addition when 0.2 µM CsA was present during fibre preparation (data not shown). Measurement of the cytochrome c content of mitochondria by Western blotting showed a 28% reduction in end-ischaemic mitochondria relative to pre-ischaemic mitochondria (Figure 3C). We conclude that loss of cytochrome c from mitochondria is not an artefact of mitochondrial preparation, and occurs independently of mPTP opening.

**Mitochondria with less cytochrome c show an increase in cytochrome c reduction state**

The mitochondrial cytochrome c content was also determined by monitoring $A_{550/540}$ in a double-beam spectrophotometer using addition of cyanide and ferricyanide to obtain fully reduced and oxidized signals, respectively. Using a value of 19.1 L.mmol$^{-1}$.cm$^{-1}$ for the reduced-oxidized extinction coefficient [24] the cytochrome c content of pre-ischaemic and end-ischaemic mitochondria was calculated to be 0.55 ± 0.02 and 0.47 ± 0.01 nmol/mg protein respectively (means ± S.E.M.; n=5; p<0.01). This represents a 15% drop following ischaemia compared to a 28% drop determined by Western blotting (Figures 3C and 3D). These measurements also revealed that cytochrome c was 20-30% more reduced in end-ischaemic mitochondria than pre-ischaemic mitochondria in all respiratory states employed (Figures 4A and 4B). We conclude that a decrease in the total content of cytochrome c is responsible for its more reduced redox state.

**End-ischaemic mitochondria produce more H$_2$O$_2$ in State 3.5 than pre-ischaemic mitochondria**

In the presence of an ADP-regenerating system to mimic physiological ATP turnover (State 3.5), both pre-ischaemic and end-ischaemic mitochondria showed the same rates of respiration in the absence of exogenous cytochrome c (Figure 5A). This contrasts with the decrease in maximal rates of respiration (State 3) observed in end-ischaemic mitochondria compared to pre-ischaemic mitochondria (Figure 3A). Under such State 3 conditions rates of respiration by end-ischaemic mitochondria were restored to pre-ischaemic values by the addition of cytochrome c whereas in State 3.5 the addition of cytochrome c increased rates of respiration by end-ischaemic mitochondria to values greater than pre-ischaemic mitochondria (Figure 5A). We did not explore the reason for the higher rates of State 3.5 respiration in end-ischaemic mitochondria supplemented with cytochrome c any further, but it is consistent with the observed increase in matrix volume of end-ischaemic mitochondria that is known to stimulate State 3 respiration [18]. Such an effect should not influence H$_2$O$_2$ production under State 3.5 conditions since this was found to be independent of the rate of mitochondrial respiration (Figure 2A). However, the data of Figure 5B show that H$_2$O$_2$ production in State 3.5 was increased two-fold in end-ischaemic mitochondria and that this increase was reversed by addition of increasing concentrations of cytochrome c (10, 25, and 50µM). For pre-ischaemic mitochondria cytochrome c addition had a much smaller effect on H$_2$O$_2$ production such that rates became similar for both control and end-ischaemic mitochondria. This effect in pre-ischaemic mitochondria is consistent with a slight permeabilization of the outer membrane occurring during mitochondrial isolation as discussed above. Overall, we conclude that a major determinant of mitochondrial H$_2$O$_2$ production in State 3.5 is the total content of cytochrome c in the IMS and that its loss during ischaemia may be responsible for the greater ROS production.
Cytochrome c redox state also affects mitochondrial H$_2$O$_2$ production

The effect of cytochrome c reduction state on H$_2$O$_2$ production was further investigated by incubating mitochondria under State 3.5 conditions with increasing micromolar concentrations of KCN. A range of 1 µM to 1.5 mM KCN gave a progressive decrease in State 3.5 respiration that was similar in pre-ischaemic and end-ischaemic mitochondria (Figure 6A). In parallel we measured the effects of the same concentrations of KCN on the amount of oxidized cytochrome c (Figure 6B), mitochondrial H$_2$O$_2$ emission (Figure 6C), and NAD(P)H autofluorescence (Figure 6D). As [KCN] increased from 1 to 10 µM the content of oxidized cytochrome c decreased to a greater extent in end-ischaemic than in pre-ischaemic mitochondria (Figure 6B). This was accompanied by an increase in the rate of H$_2$O$_2$ emission only in end-ischaemic mitochondrial (Figure 6C), although NAD(P)H autofluorescence increased to the same extent in both mitochondrial populations (Figure 6D). In the absence of KCN, end-ischaemic mitochondria showed a significantly lower NAD(P)H redox state which is discussed further in the Discussion section. At higher [KCN], where the protonmotive force collapsed, the content of oxidized cytochrome c decreased further in both pre-ischaemic and end-ischaemic mitochondria and this was accompanied by an abrupt drop in ROS production and further increase in NAD(P)H fluorescence. In Figure 7 we re-plot the data for the content of oxidized cytochrome c at low [KCN] (0-10 µM) against the rates of oxygen consumption (Panel A), H$_2$O$_2$ emission (Panel B) and NAD(P)H autofluorescence (Panel C). For NAD(P)H autofluorescence and rates of respiration the correlation with oxidized cytochrome c was different for pre-ischaemic and end-ischaemic mitochondria whereas for H$_2$O$_2$ emission the data appear to fall on the same line for both sets of mitochondria. This is consistent with the greater H$_2$O$_2$ emission observed in end-ischaemic mitochondria being the result of less oxidized cytochrome c, and hence less effective detoxification of superoxide in the IMS, rather than being caused by a change in NAD(P)H redox state. Indeed, pre-ischaemic mitochondria showed an increase in NAD(P)H autofluorescence that was not paralleled by any increase in H$_2$O$_2$ emission (Figure 7C).

Cytochrome c loss from end-ischaemic mitochondria is associated with hexokinase 2 dissociation and BcL-xL depletion

The mitochondrial content of several pro-apoptotic proteins that might be responsible for cytochrome c release from mitochondria during ischaemia was determined by Western blotting in density-gradient purified mitochondria from pre-ischaemic and end-ischaemic hearts. The end-ischaemic mitochondria showed no change in the ratio Bid / t-Bid, or P-Bad / Bad (Supplementary Figure S3 panels A and B). Nor were changes in Bax or Bak detected (Supplementary Figure S3, panels C and D). However, total hexokinase (HK) activity was decreased in end-ischaemic mitochondria (Figure 8A), and Western blotting revealed this was mainly due to a loss of HK2 (Figure 8B). End-ischaemic mitochondria were also characterized by a 63% depletion of the anti-apoptotic protein BcL-xL (Figure 8C).

DISCUSSION

The data we present in this paper provide strong evidence that both the amount of cytochrome c in the IMS and its redox state are major determinants of mitochondrial ROS production under physiological conditions and may account for the increased ROS production in ischaemia and reperfusion. We further suggest that increased cytochrome c reduction during ischaemic pre-conditioning (IP) may also provide an explanation for the mitochondrial production of ROS during IP that play a key signaling role in mediating cardioprotection. Figure 9 provides a scheme summarizing our proposals.

The sites of mitochondrial superoxide production under physiological conditions

In order to assess the (patho)physiological role of cytochrome c in ROS production it is important to understand how isolated mitochondria produce superoxide in experimental conditions that better reflect their bioenergetic state in vivo as opposed to the State 4 conditions used by many workers. State 4 is not a physiological bioenergetic state and is likely to favor superoxide production through complex I, with a minimal contribution of complex III [2]. Indeed, we found that when mitochondria were incubated in this highly
reduced state induction of cytochrome c loss by digitonin had no impact on the overall \( \text{H}_2\text{O}_2 \) production (Supplementary Figure S2A) in agreement with the data of others (see [2] for review). Nor did we observe (unpublished data) any differences in \( \text{H}_2\text{O}_2 \) production between pre-ischaemic and end-ischaemic mitochondria under State 4 conditions in the presence of NAD-linked respiratory substrates alone (i.e. glutamate + malate). This probably reflects the fact that the majority of the superoxide is produced within the matrix under these conditions and thus independent of the cytochrome c redox state. However, in our experiments mitochondrial respiration was stimulated by an ADP-regenerating system to produce an NADH:NAD\(^+\) ratio and protonmotive force (\( \Delta p \)) that are relatively low, and rate of respiration quite high compared to State 4 conditions. Under these more physiological conditions we did observe an increase in \( \text{H}_2\text{O}_2 \) production induced by cytochrome c loss (Figure 2C), suggesting that a significant amount of the superoxide is now produced in the IMS, most probably from complex III. Consistent with this, when antimycin A was added to stimulate superoxide production into the IMS from complex III [2], cytochrome c loss induced by digitonin caused a further increase in \( \text{H}_2\text{O}_2 \) production that could be reduced by addition of exogenous cytochrome c (Supplementary Figure S2B). Overall, we propose that when mitochondria are performing oxidative phosphorylation at physiological rates, a significant fraction of the superoxide produced by complex III in the IMS is buffered by the remaining oxidized cytochrome c. This hypothesis is summarized schematically in Figure 9.

**A decrease in oxidized cytochrome c in the IMS enhances mitochondrial \( \text{H}_2\text{O}_2 \) production**

Oxidized cytochrome c can scavenge superoxide in the inter-membrane space without its conversion to hydrogen peroxide that occurs spontaneously, but is enhanced by superoxide dismutase (SOD). Scavenging superoxide independently of \( \text{H}_2\text{O}_2 \) production has the advantage of avoiding the risk of hydroxyl radical formation through the Fenton reaction [2]. Hence, cytochrome c loss during ischaemia will exert additional pressure on other ROS scavenging pathways leading to increased \( \text{H}_2\text{O}_2 \) production and hydroxyl radical formation and depletion of reduced glutathione, all of which have been observed following ischaemia and reperfusion [27]. Our data show that the 15-30\% loss of cytochrome c from the IMS in ischaemia (Figure 3D) leads to an increase in the reduction state of the remaining cytochrome c (Figure 4) which then becomes unavailable to scavenge superoxide produced in the IMS resulting in greater \( \text{H}_2\text{O}_2 \) production (Figure 5B). In addition, cytochrome c loss will restrict electron flow from complexes I-III through complex IV to oxygen, potentially increasing the reduction state of NADH and flavoproteins in complex I with a consequent stimulation of matrix superoxide production [2]. However, our data show that the NAD(P)H pool was actually more oxidized in end-ischaemic mitochondria, despite mitochondrial oxygen consumption being identical in both populations. This may reflect a decrease in NADPH rather than NADH which would be consistent with the depletion of reduced glutathione observed during ischaemia [27-28]. Within the matrix detoxification of \( \text{H}_2\text{O}_2 \) is performed by glutathione peroxidase and produces oxidized glutathione that is reduced again by NADPH-mediated by glutathione reductase [2]. Thus the end-ischaemic mitochondria, which are less able to remove superoxide using cytochrome c, will produce more \( \text{H}_2\text{O}_2 \) which can enter the matrix and oxidize the reduced glutathione pool leading to depletion of NADPH. This conclusion is further supported by the demonstration that short exposure of mitochondria to \( \text{H}_2\text{O}_2 \) leads to greater subsequent \( \text{H}_2\text{O}_2 \) production [29] and provides an explanation for ROS-induced ROS release [30]. Overall, the net ROS production in both the IMS and matrix will reflect the balance between ROS production and ROS removal [31], and cytochrome c depletion has the potential to increase the former and decrease the latter. These combined effects may also account for the increase in ROS production that can follow cytochrome c release during apoptosis [32-33].

**The importance of cytochrome c loss during ischaemia for reperfusion injury**

Our data also support the hypothesis that the loss of cytochrome c from the IMS that occurs during ischaemia may be responsible, at least in part, for the oxidative stress that, together with calcium overload, is the major trigger of mPTP opening during reperfusion. This is a major cause of reperfusion injury [12, 34] and inhibition of mPTP opening with cyclosporine...
A or sanguinoferrin A protects hearts from reperfusion injury in a variety of settings, including isolated perfused hearts, animal models and patients undergoing angioplasty [12, 34-35]. The importance of ROS in mediating mPTP opening during reperfusion is supported by the ability of MitoQ, a mitochondrial-targeted ROS scavenger, to provide potent cardioprotection [36]. Furthermore, other well-established cardioprotective protocols such as ischaemic pre-conditioning (IP), post-conditioning and temperature pre-conditioning (TP) are also associated with a decrease in oxidative stress during ischaemia and reperfusion that can explain the observed inhibition of mPTP opening [12, 34, 37-39].

Nitric oxide is a naturally occurring inhibitor of cytochrome oxidase that has been implicated in cardioprotection but may also, through peroxynitrite formation, act as an inducer of mPTP opening and reperfusion injury [40]. We have considered whether NO might also mimic the actions of cyanide and lead to an increase in the reduction state of cytochrome c with a resulting loss of ROS scavenging. We tested this possibility by using the NO donor diethylamine NONOate diethylammonium salt (DEA) at concentrations between 2.5 and 10 µM. These concentrations of DEA caused a inhibition of State 3.5 respiration similar to those cyanide concentrations that induced increased H$_2$O$_2$ production in ischaemic mitochondria (Supplementary Fig. S5 panel A). However, unlike cyanide the presence of DEA decreased rather than increased H$_2$O$_2$ production under all conditions tested (Supplementary Fig. S5 panel B). Since NO rapidly reacts with superoxide to produce peroxynitrite [40] it is likely that the decrease in H$_2$O$_2$ production reflects the removal of superoxide in this way rather than a decreased superoxide production. Consistent with this we have confirmed that the presence of similar concentrations of DEA also decreased the production of H$_2$O$_2$ by purified xanthine oxidase in the presence of SOD (Supplementary Fig. S6). Since peroxynitrite is itself an inducer of mPTP opening [40], any protection against mPTP opening that might be offered by NO removing superoxide is likely to be offset by the increase in peroxynitrite.

**The mechanism of cytochrome c release in ischaemia**

The mPTP is not involved

The loss of cytochrome c during ischaemia is unlikely to be an artefact of mitochondrial preparation since we observed the same effect in saponin-permeabilized fibres (Figure 3B). Nor do our data suggest that opening of the mPTP during ischaemia is responsible since pre-treatment of hearts with CsA (Figure 3) and the presence of CsA during the preparation of fibres (data not shown) were both without effect. These data differ from those of Borutaite et al who observed similar cytochrome c loss but found it to be CsA–sensitive [15]. We cannot explain this discrepancy, but our data are consistent with those of Morin et al [41] who showed that in vivo pre-treatment of rats with CsA did not block cytochrome c release from liver mitochondria that occurs during ischaemia. Furthermore, the data presented here are in agreement with our previous studies using mitochondrial entrapment of $[^3]$H-2-deoxyglucose-6-phosphate to detect mPTP opening in situ. In these studies we observed mPTP opening during reperfusion but not during ischaemia [42].

Cytochrome c loss does not lead to caspase activation during ischaemia

It might be predicted that the release of cytochrome c during ischaemia should induce procaspase 3 cleavage and activation as was reported by Borutaite et al [15]. However, we were unable to detect any cleaved pro-caspase 3 in freeze-clamped hearts following 30 minutes of ischaemia (Supplementary Figure S4, panel A). We did detect increased cleavage of the DEVD peptide in cytosolic extracts of ischaemic hearts when compared to pre-ischaemic samples, but this was completely insensitive to the caspase 3 inhibitor DEVD-CHO which totally blocked the activity of recombinant caspase 3 (Supplementary Figure S4, panel B). Other groups have also been unable to detect caspase 3 activation following ischaemia and reperfusion [43]. Furthermore, it is difficult to reconcile significant caspase activation during 30 min ischaemia with cardioprotection induced at reperfusion by post-conditioning [44]. Calpain activation has been demonstrated during ischaemia and reperfusion and calpain inhibition significantly decreases infarct size [45-47]. Thus the ischaemia-induced cleavage of the DEVD peptide might be secondary to calpain activation induced by the elevated cytosolic [Ca$^{2+}$] that accompanies ischaemia. However, more
recently Hernando et al reported that although ischaemia induced m-calpain translocation to the membrane, this was not associated with an increase in its basal activity [48].

A role for hexokinase 2 and Bcl-x

It has been proposed that HK2 binding to mitochondria plays an anti-apoptotic role and inhibits mPTP opening [17, 49]. HK2 is over-expressed in cancer cells and several studies have shown a correlation between the amount of bound mitochondrial HK2 and the ability of mitochondria to resist apoptotic stimuli [17]. Indeed, it has been proposed that HK2 detachment may be directly or indirectly responsible for the recruitment or activation of pro-apoptotic proteins responsible for OMM permeabilization that leads to the loss of cytochrome c and other apoptogenic factors [17]. Our data confirm those of others [50] that ischaemia decreases HK2 binding to mitochondria (Figure 8) suggesting a possible role for this protein in the observed cytochrome c release. What causes this dissociation during ischaemia is not known, but it could reflect the increased glucose-6-phosphate concentrations that occur during ischaemia [51]. Glucose-6-phosphate is a potent non-competitive inhibitor of HK2 whose binding to the enzyme causes its dissociation from the OMM [17]. The binding partner for HK2 in the OMM was thought to be the voltage dependent anion channel (VDAC) and evidence has been presented for regulation of binding through VDAC1 phosphorylation [49]. Pro-survival signaling pathways such as those mediated by Akt phosphorylation and inhibition of glycogen synthase kinase 3 (GSK3β) increase HK2 binding to mitochondria as a critical part of their anti-apoptotic mechanism [17]. Interestingly, these same pathways have been implicated in the protection of hearts by ischaemic preconditioning [52] which is known to prevent the loss of HK2 binding to mitochondria during ischaemia [50].

Thus it seems possible that many of the signaling pathways implicated in preconditioning could target HK2 binding, preventing its dissociation during ischaemia. This would then prevent cytochrome c loss and lead to less ROS production, less oxidative stress and hence avert the sensitization of the mPTP to calcium that occurs during reperfusion. The mechanism we propose would also allow for the observed role of glycogen depletion in ischaemic preconditioning [53] since glycogen is the source of the glucose-6-phosphate that accumulates in ischaemia and can enhance HK2 dissociation. We propose that regulation of cytochrome c loss in this way provides a unifying model for how different pre-conditioning protocols may act to reduce the mitochondrial oxidative stress during reperfusion leading to less mPTP opening and necrotic cell death. Furthermore, this mechanism does not require the migration of protein kinases across the inner and outer mitochondrial membranes as suggested by some [52] but which we could not observe [37]. It is unlikely that HK2 loss directly causes release of cytochrome c, but is more likely to reflect or mediate a change in the mitochondrial content of members of the Bcl2 family that have been implicated in this release process. Although we could not detect a change in Bad, Bax, Bak and Bid (Supplementary Figure S3) we did detect a decrease in the content of the anti-apoptotic protein Bcl-xL (Fig 8). In this context it should be noted that Bcl-xL over-expression has been reported to reduce hypoxia induced ROS production and cell death in PC12 cells [54] and that adenovirus-mediated Bcl-xL gene transfer protects hearts from ischaemia / reperfusion injury [55]. The decrease in Bcl-xL expression may reflect its rapid proteolysis, perhaps mediated by calpains [56], to produce a truncated form of Bcl-xL that is capable of inducing OMM permeabilization to cytochrome c [57]. This mechanism would be consistent with the powerful cardioprotective effects of calpain inhibitors [45-47, 58-59].

Cytochrome c reduction state could play a role in redox signaling

Only the oxidized form of cytochrome c scavenges superoxide and thus any condition which produces an increase in cytochrome c reduction state without greatly depolarizing mitochondria would be expected to enhance superoxide production in the inter-membrane space and subsequently hydrogen peroxide levels in the cytosol. Our data employing low concentrations of cyanide to give a modest increase in cytochrome c reduction state confirm this (Figure 7B,C). It has been shown that ischaemic preconditioning is associated with transient ROS production and that scavenging this ROS prevents its cardioprotective effects [9, 38, 60]. Indeed, an elegant study from Schumacker’s group using ROS-sensitive
targeted proteins has recently demonstrated that in vascular smooth muscle cells hypoxia leads to a decrease of ROS in the matrix but an increase in the mitochondrial intermembrane space consistent with this proposal [61]. There is also evidence that in hypoxia the regulation of gene expression through stabilization of hypoxia induced factor-1α (HIF-1α) is mediated through increased mitochondrial ROS production [62]. Since both hypoxia and transient ischaemia will cause cytochrome c reduction this may provide the mechanism for the production of the ROS that signals their effects.

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Disclosures
None

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FIGURE LEGENDS

Figure 1. Scheme summarizing the perfusion protocols used.

Figure 2. The effect of cytochrome c loss on the rates of H$_2$O$_2$ emission and oxygen consumption in state 3.5
Panel A shows the rate H$_2$O$_2$ emission as a function of oxygen consumption of control mitochondria in the presence of glutamate, malate and succinate (GMS) before and after addition of increasing concentrations of FCCP. Numbers in parentheses indicates the concentrations in nmol / L used. Panel B, shows oxygen consumption of control mitochondria in the presence of GMS, 5mM creatine and 40 µg creatine kinase monitored after addition of 200 µM ATP in the absence or presence of 10 µM cytochrome c (+Cc). In two parallel groups mitochondria were treated with 0.72 mg digitonin per mg mitochondria (Digito) or by 5 µM KCN (KCN) before addition of ATP. Panel C, shows the corresponding rate of H$_2$O$_2$ emission, monitored as described in the “Materials and Methods” section, for the 3 groups of mitochondria shown in panel B. Panel D, shows the corresponding NAD(P)H autofluorescence, monitored as described in the “Materials and Methods” section. The minimum and maximum values were obtained after addition of 1 µM FCCP and 1.5 mM KCN, respectively. All data are given as means ± S.E.M. of 5-10 separate preparations for each group. Panel B: * p<0.001 vs. (a); ** p<0.001 vs. (a). Panel C: * p<0.001 vs. (a). Panel D: * p<0.02 vs. (a), ** p<0.001 vs. (a).

Figure 3. The effect of ischaemia on the maximal rate of mitochondrial respiration and cytochrome c content.
Panel A shows the rate of oxygen consumption of pre-ischaemic (Pre) and end-ischaemic (Isch) mitochondria in presence of GMS (solid bars), and after addition of 1.5 mM ADP in the absence (ADP, open bars) or presence of 10 µM cytochrome c (Cc, grey bars). In Panel B, permeabilized fibres were prepared from Pre and Isch hearts perfused in the absence or presence of 0.2 µM cyclosporine A (Isch+CsA) and rates of oxygen consumption determined in presence of GMS after addition of 200 µM ATP in absence (ATP, solid bars) or presence of 10 µM cytochrome c (Cc, grey bars), and after addition of 10 µg oligomycin + 5 µM carboxyatractylloside (S4, open bars). Panel C and D, shows representative Western blots and mean data for the ratio of cytochrome c to adenine nucleotide translocase (ANT) for Pre and Isch hearts perfused in the absence or presence of 0.2 µM cyclosporine A (Isch+CsA). Numbers in parentheses represent the absolute difference between the respiration coupled to ATP synthesis measured in the presence and absence of cytochrome c. All data are given as means ± S.E.M. of 5 (permeabilized fibres) to 16 (isolated mitochondria) separate preparations for each group. Panel A: * p<0.002 vs. (a); ** p<0.003 vs. (b). Panel B: * p<0.008 vs. (a). Panel D: # p<0.03 vs. (a).

Figure 4. The effect of ischaemia on the redox state of cytochrome c.
Mitochondria were incubated in KCl buffer containing GMS plus 5mM creatine and creatine kinase (160 µg / mg protein). Panel A shows representative traces of cytochrome c reduction of pre-ischaemic (black, circles) and end-ischaemic (grey, circles) mitochondria as a function of time when measured at 550-540 nm with additions made as indicated: 200 µM ATP; 15 nM FCCP; 1.5 mM KCN; 3.5 mM FeK$_3$(CN)$_6$; 7 mM Na$_2$S$_2$O$_4$. Data were normalized to fully oxidized (FeK$_3$(CN)$_6$) and fully reduced (Na$_2$S$_2$O$_4$) values. Panel B presents mean data (± S.E.M.; n=5) for % of reduced cytochrome c in pre-ischaemic (Pre, solid bars) and end-ischaemic (Isch, grey bars) mitochondria in the presence of GMS alone or after addition of ATP or FCCP.

Figure 5. The effect of ischaemia on rates of oxygen consumption and H$_2$O$_2$ production in state 3.5.
Mitochondria were incubated in KCl buffer supplemented with GMS, 5 mM creatine and 40 µg creatine kinase as described in the “Materials and Methods” section. Panel A presents data for the oxygen consumption of pre-ischaemic (Pre) and end-ischaemic (Isch)
mitochondria monitored before (solid bars) and after addition of 200 µM ATP in the absence (open bars) or presence (gradient grey bars) of 10, 25 or 50 µM cytochrome c. Numbers in parentheses represent the absolute difference between the rates of respiration coupled to ATP synthesis measured in the presence and absence of cytochrome c. Panel B presents data for the rate of H$_2$O$_2$ production for the same groups monitored after addition of 200 µM ATP in the absence (open bars) or presence (gradient grey bars) of 10, 25 and 50 µM cytochrome c. All data are means ± S.E.M. of 5 separate mitochondrial preparations for the two groups. Panel A: * $p<0.05$ vs. (a), ** $p<0.0001$ vs. (b). Panel B: * $p<0.0001$ vs. (a), ** $p<0.0001$ vs. (b).

Figure 6. The effects of increasing [KCN] on mitochondrial function.
Mitochondria were incubated in KCl buffer supplemented with GMS, 5 mM creatine + creatine kinase (160 µg / mg protein). After addition of 200 µM ATP, progressive additions of KCN were made (1 µM to 1.5 mM). Panel A shows mean data (± S.E.M.; n=5) for the oxygen consumption of pre-ischaemic (Pre, solid circles) and end-ischaemic (Isch, grey circles) mitochondria. Panels B-D show parallel data for oxidized cytochrome c, H$_2$O$_2$ production, and NAD(P)H autofluorescence, respectively. For NAD(P)H fluorescence (panel D) values were normalized to the value obtained in the Pre group without KCN after addition of 1 µM FCCP and 1.5 mM KCN respectively. Values for 100% reduction, obtained after addition of 1.5 mM KCN, were 36.4 ± 0.4 and 35.9 ± 0.8 ($p = 0.63$) for pre-ischaemic and end-ischaemic mitochondria respectively. Data for the 2 groups are presented as means ± S.E.M. for 5 separate mitochondrial preparations with bars indicating p values Isch vs. Pre.

Figure 7. The relationship between H$_2$O$_2$ emission, oxygen consumption, NAD(P)H fluorescence and the content of oxidized cytochrome c.
Pre-ischaemic (open circles) and end-ischaemic (grey circles) data obtained at low [KCN] (0 to 1 µM) for oxygen consumption (panel A), H$_2$O$_2$ emission (panel B), NAD(P)H autofluorescence (panel C), were taken from Figure 6 and replotted as a function of the total content of oxidized cytochrome c.

Figure 8. Effects of ischaemia on the mitochondrial content of hexokinase and Bcl-xL.
Panel A shows hexokinase specific activity (means ± S.E.M.; n=6) normalized to citrate synthase activity as described in Methods. HK specific activity was 58.7 ± 2.7 and 37.3 ± 3.8 nmole / min / mg protein in Pre and Isch mitochondria, respectively. Panel B top panel shows a typical Western blot used to determine the mitochondrial content of HK1, HK2 and ANT and the bottom panel presents mean data (± S.E.M. n=10) for the ratio of HK1 and HK2 to ANT obtained by scanning blots. * $p<0.0001$ Isch HK 2 vs. Pre HK 2. Panel C top panel shows a typical Western blot used to determine the mitochondrial content of Bcl-xL and the bottom panel, mean data (± S.E.M. n=5) for the ratio of Bcl-xL to ANT obtained by scanning blots. * $p<0.003$ Isch Bcl-xL vs. Pre Bcl-xL.

Figure 9. Scheme illustrating the role of oxidized cytochrome c in mitochondrial ROS production.
In this scheme mitochondria are shown under conditions in which they are making ATP (State 3.5) and thus are characterized by a lower proton motive force and NADH/NAD$^+$ ratio and higher rate of oxygen consumption than in State 4. Ischaemia decreases the available oxidized cytochrome c in the inter-membrane space, thus impairing its scavenging ability and increasing the net rate of H$_2$O$_2$ production by mitochondria. The increased H$_2$O$_2$ can diffuse into the matrix thus challenging the detoxification system (blue lines) and further enhancing ROS production. ROS production may eventually lead to mPTP sensitization to calcium through oxidation of matrix facing-thiol proteins. In this diagram no assumptions have been made regarding the site of the electron transport chain that could be responsible for superoxide production (red lines). We propose that hypoxia and transient ischaemia (as in ischaemic preconditioning) may modulate cytochrome c redox state leading to increased superoxide production in the inter-membrane space and formation of H$_2$O$_2$ that activates signaling pathways.
Fig. 1

- Normoxic Perfusion
- Global Ischaemia
- CsA 0.2µM

Time (min)
Fig. 2

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

I. 

J. 

K. 

L. 

M. 

N. 

O. 

P. 

Q. 

R. 

S. 

T. 

U. 

V. 

W. 

X. 

Y. 

Z. 

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Fig. 3

A
Oxygen consumption (nmol O$_2$/min/mg protein)

GMS ADP Cc
Pre

(63±13) (227±13) ** p<0.05

GMS ADP Cc
Isch

B
Oxygen consumption (nmol O$_2$/min/mg dry weight)

ATP Cc S4
Pre

(0.3±0.2) (6.1±1.0) (5.6±0.8) ** p<0.001

ATP Cc S4 +CsA
Isch

C
Cyt c →
ANT →

D
Relative band intensity

Pre

(+) * p<0.001

+CsA
Isch

* p<0.05

# p<0.001

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Fig. 4

A. Time course of the percentage of reduced cytochrome c in Mito. The graph shows the effect of various compounds on the percentage of reduced cytochrome c over time. The compounds include Mito, FeK₃(CN)₆, KCN, and Na₂S₂O₄.

B. Bar graph showing the percentage of reduced cytochrome c under different conditions. The conditions are Pre, Isch, GMS, ATP, and FCCP. The p-values indicate statistical significance:
- Pre vs. Isch: p=0.02
- Pre vs. Isch: p=0.0003
- Pre vs. Isch: p<0.0001

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Fig. 5

A

Oxygen consumption (nmol O$_2$/min/mg protein)

GMS ATP +Cc GMS ATP +Cc

Pre Isch

(29±5) (114±2)

B

H$_2$O$_2$ emission (pmol/min/mg protein)

ATP +Cc ATP +Cc

Pre Isch

(a) (b)

* **
Fig. 6

A. Oxygen consumption (nmol O$_2$/min/mg protein) vs. Log([KCN] µM)

B. Oxidised cytochrome c (nmol/mg protein) vs. Log([KCN] µM)

C. H$_2$O$_2$ emission (pmol/min/mg protein) vs. Log([KCN] µM)

D. NAD(P)H fluorescence (% of Pre values min and max) vs. Log([KCN] µM)
Fig. 7

A. Oxygen consumption (nmol O$_2$/min/mg protein) vs. Oxidised Cytochrome c (nmol/mg protein)

B. H$_2$O$_2$ emission (pmol/min/mg protein) vs. Oxidised Cytochrome c (nmol/mg protein)

C. NAD(P)H fluorescence (% of Pre values min and max) vs. Oxidised Cytochrome c (nmol/mg protein)
Fig. 8

A) Hexokinase activity (normalised to CS content)

B) Relative band intensity

C) Bcl-xL

HK II

HK I

ANT

HK 2

HK 1
Fig. 9

Signalling pathways

Ischemia

OMM

Inter-membrane space

H_2O_2

Cu/ZnSOD

O_2^-

O_2

Cyt c Fe^{3+}

Cyt c Fe^{2+}

H^+

H^+

NADH

NAD^+

Succ

Fum

H_2O

½O_2

H_2O_2

MnSOD

Fenton reaction

OH^-

H_2O_2

SPx

GSH

NADPH

H_2O

GSSG

NADP^+

GR

MPTP sensitization

NADH / NAD^+ low
∆p low
Respiration High
ATP synthesis High

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