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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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Oxidized cytochrome *c* is a powerful superoxide scavenger within the mitochondrial IMS (intermembrane space), but the importance of this role *in situ* has not been well explored. In the present study, we investigated this with particular emphasis on whether loss of cytochrome *c* from mitochondria during heart ischaemia may mediate the increased production of ROS (reactive oxygen species) during subsequent reperfusion that induces mPTP (mitochondrial permeability transition pore) opening. Mitochondrial cytochrome *c* depletion was induced *in vitro* with digitonin or by 30 min ischaemia of the perfused rat heart. Control and cytochrome *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. Cytochrome *c*-deficient mitochondria produced more H_2O_2 than control mitochondria, and exogenous cytochrome *c* addition reversed this increase. In the presence of increasing [KCN] rates of H_2O_2 production by both pre-ischaemic and end-ischaemic mitochondria correlated with the oxidized cytochrome *c* content, but not with rates of respiration or NAD(P)H autofluorescence. Cytochrome *c* loss during ischaemia was not mediated by mPTP opening (cyclosporine-A insensitive), neither was it associated with changes in mitochondrial Bax, Bad, Bak or Bid. However, bound HK2 (hexokinase 2) and Bcl-xL were decreased in end-ischaemic mitochondria. We conclude that cytochrome *c* loss during ischaemia, caused by outer membrane permeabilization, is a major determinant of H_2O_2 production by mitochondria under pathophysiological conditions. We further suggest that in hypoxia, production of H_2O_2 to activate signalling pathways may be also mediated by decreased oxidized cytochrome *c* and less superoxide scavenging.

Key words: Bcl-xL, cytochrome *c*, hexokinase (HK), mitochondrial permeability transition pore (mPTP), superoxide.

INTRODUCTION

ROS (reactive oxygen species) such as superoxide and H_2O_2 are known to play important signalling 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 IMS (intermembrane space), 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 H_2O_2 by the manganese (matrix) and zinc (IMS) SOD (superoxide dismutase). The H_2O_2 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 details). In the presence of ferrous ions, this pathway of superoxide removal runs the risk of generating the highly damaging hydroxyl radical from H_2O_2 through the Fenton reaction [2]. However, in the IMS, there is another potential mechanism for superoxide removal that involves its conversion back into oxygen by oxidized cytochrome *c* (see [1,3]). Cytochrome *c* is present at approx. 1 mM in the IMS and can be reduced by superoxide with a rate constant of approx. 10^7 $M^{-1} \cdot s^{-1}$ [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 the present paper, we report the results of such investigations 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 mPTP (mitochondrial permeability transition pore), 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 the present study, we explored this possibility more rigorously. The Qo site of complex III can be a major site of superoxide production into the IMS where cytochrome *c* resides [2]. Thus cytochrome *c* loss would lead to less superoxide scavenging by this route leading to greater production of H_2O_2 through SOD and hence the possibility

Abbreviations used: ANT, adenine nucleotide translocase; CHO, Chinese-hamster ovary; CsA, cyclosporine A; DEA, diethylamine NONOate diethylammonium salt; DTT, dithiothreitol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 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; ROS, reactive oxygen species; SOD, superoxide dismutase; VDAC, voltage-dependent anion channel.

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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 increases so would that of complex 1, potentially increasing matrix production of superoxide, H_2O_2 and hydroxyl radicals [2]. In the present study, we demonstrated 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 H_2O_2 production and that loss of cytochrome *c* from mitochondria during ischaemia can explain the observed increase in H_2O_2 production. This cytochrome *c* loss occurs without mPTP opening, but is associated with a loss of bound HK2 (hexokinase 2) and a depletion in Bcl-xL content that are implicated in the regulation of OMM (outer mitochondrial membrane) permeability that leads to cytochrome *c* release in apoptosis [16,17].

MATERIALS AND METHODS

Antibodies and chemicals

The antibodies against the following were used in the present study: Bax B-9 (mouse monoclonal, Santa Cruz Biotechnology), Bak (rabbit monoclonal, Abcam), Bad (rabbit monoclonal, Cell Signaling Technology) P-Bad (rabbit monoclonal, Cell Signaling Technology), Bid (goat polyclonal, R&D Systems), t-Bid (rat monoclonal, R&D Systems), HK1 (mouse monoclonal, Chemicon Milipore), HK2 (rabbit monoclonal, Cell Signaling Technology), cytochrome *c* (mouse monoclonal, BD Biosciences Pharmingen), Bcl-xL (rabbit monoclonal, Cell Signaling Technology). All chemicals used in the present study were purchased from Sigma unless otherwise stated.

Heart perfusion

All procedures conformed to the U.K. Animals (Scientific Procedures) Act 1986. Male Wistar rats (225–250 g) were stunned and killed by cervical dislocation and hearts (~0.75 g) were rapidly removed into ice-cold Krebs–Henseleit buffer containing (in mmol/l) NaCl 118, $NaHCO_3$ 25, KCl 4.8, KH_2PO_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 min treatment with 0.2 μM CsA (cyclosporine A) 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.

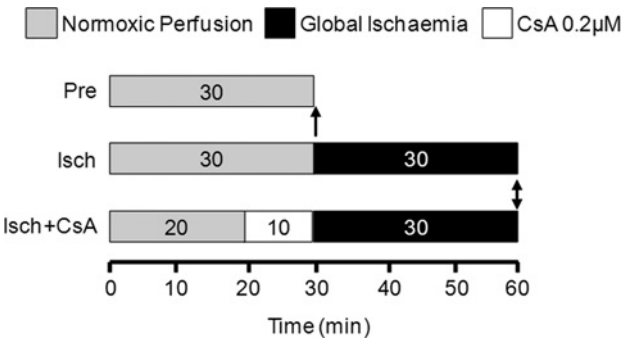

Figure 1 Summary of the perfusion protocols used

Isch, ischaemia; Pre, pre-ischaemia.

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 of isolation buffer (ISA: 300 mM sucrose, 2 mM EGTA and 10 mM 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 7500 ***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 7000 ***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 7000 ***g*** for 10 min. The final purified mitochondrial pellet was resuspended in ISA and the protein concentration determined by the Biuret method using BSA as a standard. Mitochondria were kept on ice at a final concentration of 50 mg/ml for not more than 4 h.

Polytron method

This was performed essentially as described previously [19]. Each heart was homogenized at 4 °C in 6 ml of ISA using a Polytron tissue disruptor (Kinematica) at 10 000 rev./min 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 7500 ***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/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 BCA (bicinchoninic acid)-based 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: CaK_2EGTA 2.77; K_2EGTA 7.23 (pCa = 7); $MgCl_2$ 6.56; DTT (dithiothreitol) 0.5; Mes 50; imidazole 20; taurine 20; Na_2ATP 5.3; and creatine phosphate 15. The pH was adjusted to 7.1 at room temperature with 10 M KOH.

Solution B in mmol/l: CaK_2EGTA 2.77; K_2EGTA 7.23 (pCa = 7); $MgCl_2$ 1.38; DTT 0.5; Mes 100; imidazole 20; taurine 20; and KH_2PO_4 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 of digitonin per 250 μg of 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 instrument. Unless stated otherwise the respiratory substrate was a mixture of GMS (5 mM L-glutamate + 2 mM L-malate + 5 mM succinate) 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 of 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 the legends to Figures 2–5) after ADP or ATP + creatine.

Measurement of H_2O_2 production and NAD(P)H autofluorescence

The rate of H_2O_2 production was determined with the fluorescent H_2O_2 indicator, Amplex Red (30 μM), in the presence of peroxidase using λ_{ex} and λ_{em} wavelengths of 540 and 585 nm respectively. Measurements were made under State 3.5 conditions (identical with 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 H_2O_2 production was calibrated using a standard curve generated under the same experimental conditions with additions of 0–40 pmol of exogenous H_2O_2 (see Supplementary Figure S6 available at http://www.BiochemJ.org/bj/436/bj4360493add.htm). It was confirmed that the addition of exogenous SOD, to ensure conversion of superoxide into H_2O_2, was without effect on rates of H_2O_2 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 Figure S1 available at http://www.BiochemJ.org/bj/436/bj4360493add.htm). Indeed under these conditions exogenous cytochrome *c* gave a substantial reduction in H_2O_2 production (Supplementary Figure S2B available at http://www.BiochemJ.org/bj/436/bj4360493add.htm). Taken together these results suggest that, normally, rates of superoxide production in the IMS are sufficiently low for complete dismutation to H_2O_2 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 H_2O_2, which is accelerated by added SOD. This superoxide can also be removed by exogenous cytochrome *c* leading to a reduction in the H_2O_2 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 (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) 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 (*A*) at 550 nm occurring upon cytochrome *c* reduction for any non-specific light-scattering changes that are mirrored in the change in *A* 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 of standard KCl buffer under different respiratory conditions and $A_{550\text{-}540}$ monitored continuously. Additions were made through an injection port as required concluding with 3.5 mM $FeK_3(CN)_6$ followed by 7 mM $Na_2S_2O_4$ to determine the fully oxidized and reduced $A_{550/540}$ respectively. The molar absorption coefficient (ε) for reduced minus oxidized cytochrome *c* at this wavelength pair was taken as $19.1\,l \cdot mmol^{-1} \cdot cm^{-1}$ [24].

Measurement of cytochrome *c*, Bax, Bak, Bad/P-Bad, Bid/t-Bid, Bcl-xL and HK by Western blotting

Mitochondria and cytosolic fractions, prepared by the polytron method (see above), were separated by SDS/PAGE (12 % gel for cytochrome *c* and Bcl-xL, 10 % gel for Bak and Bax and 5 % gel for HK) 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 were developed using the required Ig HRP (horseradish peroxidase) secondary antibody, with ECL/ECL + detection (Amersham Biosciences). 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 blots, and AlphaEase v5.5 software to analyse band intensities. 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 ANT (adenine nucleotide translocase).

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 analysed 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 Technology). Band intensities were normalized using ANT and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) for the mitochondrial and cytosolic samples respectively. Caspase 3 activity was determined using a CASP3 kit (Sigma–Aldrich) according to the manufacturer's instructions. This assay detects cleavage of the caspase 3 substrate Ac-DEVD (*N*-acetyl-Asp-Glu-Val-Asp)-*p*-nitroaniline by measuring the *A* (405 nm) of the *p*-nitroaniline product. Samples of the cytosolic fraction obtained from the frozen heart powder (see above), were incubated at 0.5 mg of protein/ml in the assay buffer at 37 °C and the formation of *p*-nitroaniline measured every 30 min for 3 h. 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 *A* of known concentrations of *p*-nitroaniline.

Measurement of HK, citrate synthase and complex I specific activity

Aliquots (0.75 mg of 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 KH_2PO_4 and 50 μM DTT (pH 7.2). For assay of HK, samples (20, 30 or 40 μl) were added to 1 ml of assay buffer (pH 7.4) containing 100 mM Tris/HCl, 0.4 mM $NADP^+$, 10 mM $MgCl_2$, 5 mM ATP, 0.3 % Triton X-100 and 0.5 unit/ml of G6PDH (glyceraldehyde-6-phosphate dehydrogenase) and incubated for 2 min at 37 °C before addition of glucose (1 mM final) to start the reaction. HK 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

Results are presented as means $\pm$ S.E.M. Statistical significance was evaluated using one-way ANOVA (Kaleidagraph, 4.03), and differences were considered significant at $P < 0.05$.

RESULTS

Loss of cytochrome *c* increases mitochondrial H_2O_2 production

In order to study the relationship between H_2O_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 protonmotive force (Δp) and the ratio NADH to NAD^+ [2]. Consequently, H_2O_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 2(A), the rate of H_2O_2 production was decreased linearly as mitochondrial respiration increased from basal to 270 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, and thereafter showed no significant decrease as respiration was increased from 270 to 380 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$. 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 $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ 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 μg/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 H_2O_2 production were determined in State 3.5. It should be noted that under these more physiological conditions rates of H_2O_2 production were only approx. 12 % of those in State 4 used in the majority of published studies on the regulation of mitochondrial ROS production (Supplementary Figure S1). After addition of digitonin, mitochondrial respiration was decreased by 14 %, and H_2O_2 production increased by 45 % when compared with corresponding controls (Figures 2B and 2C). After addition of 10 μM 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 H_2O_2 emission in State 3.5, 5 μM potassium cyanide was added to mimic the effects of digitonin on both the rate of oxygen consumption and NAD(P)H autofluorescence (Figures 2B and 2D). 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 H_2O_2 production (Figure 2C). We conclude that loss of cytochrome *c* from mitochondria increases H_2O_2 emission. Note that even in control mitochondria, addition of exogenous cytochrome *c* was able to decrease H_2O_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 H_2O_2 and detection by Amplex Red.

Mitochondrial loss of cytochrome *c* during ischaemia occurs without mPTP opening

Hearts were perfused $\pm$ 0.2 μM CsA prior to ischaemia and mitochondria isolated either before (Pre) or after Isch (ischaemia; 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 μM 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 min 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 $\pm$ 2 % respectively, whereas the equivalent stimulation in isolated mitochondria was 8 $\pm$ 1 % and 32 $\pm$ 2 % respectively;

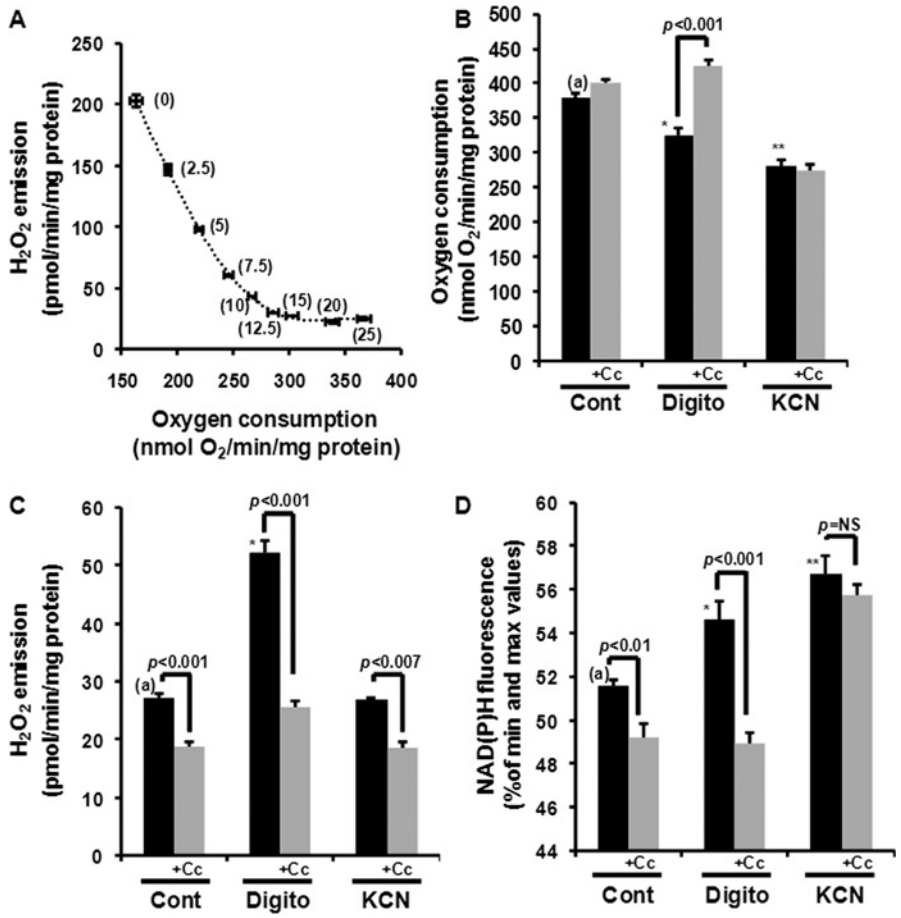

Figure 2 The effect of cytochrome *c* loss on the rates of H_2O_2 emission and oxygen consumption in State 3.5

(**A**) Rate of H_2O_2 emission as a function of oxygen consumption of control mitochondria in the presence of GMS before and after addition of increasing concentrations of FCCP. Numbers in parentheses indicate the concentrations in nmol/l used. (**B**) Oxygen consumption of control mitochondria in the presence of GMS, 5 mM 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 of digitonin per mg of mitochondria (Digito) or by 5 μM KCN before addition of ATP. (**C**) Corresponding rate of H_2O_2 emission, monitored as described in the Materials and methods section, for the three groups of mitochondria shown in (**B**). (**D**) 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 results are given as means $\pm$ S.E.M. of five to ten separate preparations for each group. (**B**) $^*P < 0.001$ compared with (a); $^{**}P < 0.001$ compared with (a). (**C**) $^*P < 0.001$ compared with (a). (**D**) $^*P < 0.02$ compared with (a), $^{**}P < 0.001$ compared with (a).

in both cases the increase caused by ischaemia was approx. 25 %. Leak of cytochrome *c* 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 (results 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 $19.1\,l \cdot mmol^{-1} \cdot cm^{-1}$ for the reduced-oxidized molar absorption coefficient (ε) [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 $\pm$ S.E.M., $n = 5$, $P < 0.01$). This represents a 15 % fall following ischaemia compared with a 28 % fall 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_2O_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

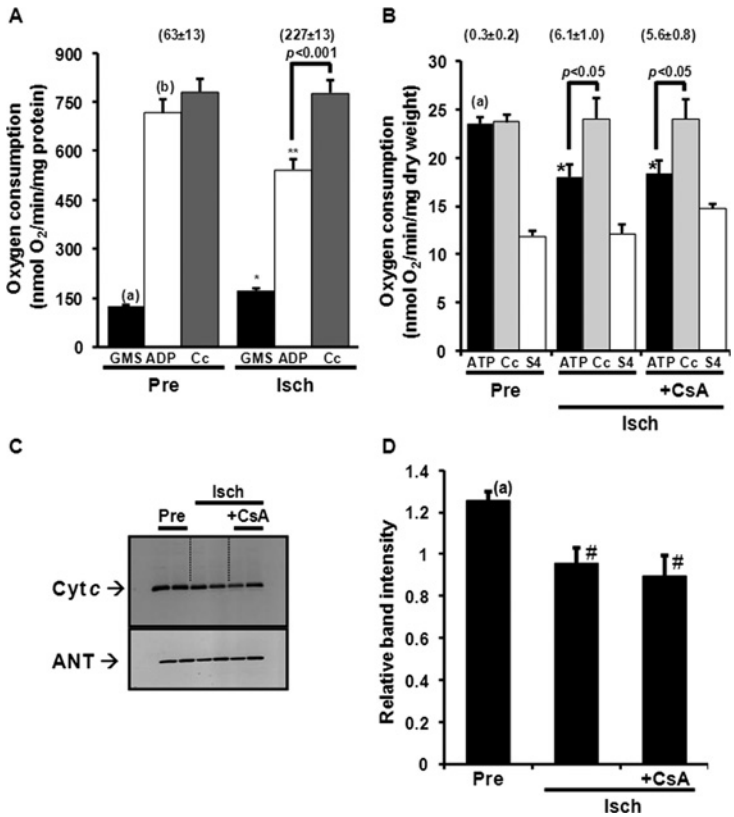

Figure 3 The effect of ischaemia on the maximal rate of mitochondrial respiration and cytochrome *c* content

(**A**) Rate of oxygen consumption of pre-ischaemic (Pre) and end-ischaemic (Isch) mitochondria in the presence of GMS (black bars), and after addition of 1.5 mM ADP in the absence (ADP, white bars) or presence of 10 μM cytochrome *c* (Cc, grey bars). (**B**) Permeabilized fibres were prepared from Pre and Isch hearts perfused in the absence or presence of 0.2 μM CsA (Isch + CsA) and rates of oxygen consumption determined in the presence of GMS after addition of 200 μM ATP in the absence (ATP, black bars) or the presence of 10 μM cytochrome *c* (Cc, grey bars), and after addition of 10 μg oligomycin + 5 μM carboxyatractyloside (S4, white bars). (**C** and **D**) Representative Western blots and mean data for the ratio of cytochrome *c* to ANT for Pre and Isch hearts perfused in the absence or presence of 0.2 μM CsA (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 results are given as means ± S.E.M. of five (permeabilized fibres) to 16 (isolated mitochondria) separate preparations for each group. (**A**) $*P < 0.002$ compared with (a); $**P < 0.003$ compared with (b). (**B**) $*P < 0.008$ compared with (a). (**D**) $\#P < 0.03$ compared with (a).

contrasts with the decrease in maximal rates of respiration (State 3) observed in end-ischaemic mitochondria compared with 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 the 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_2O_2 production under State 3.5 conditions since this was found to be independent of the rate of mitochondrial respiration (Figure 2A). However, the results of Figure 5(B) show that H_2O_2 production in State 3.5 was increased 2-fold in end-ischaemic mitochondria and that this increase was reversed by the addition of increasing concentrations of cytochrome *c* (10, 25 and 50 μM). For pre-ischaemic mitochondria, cytochrome *c* addition had a much smaller effect on H_2O_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_2O_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_2O_2 production

The effect of cytochrome *c* reduction state on H_2O_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_2O_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_2O_2 emission only in end-ischaemic mitochondria (Figure 6C); although NAD(P)H autofluorescence increased to the same extent in both mitochondrial populations (Figure 6D). In the absence

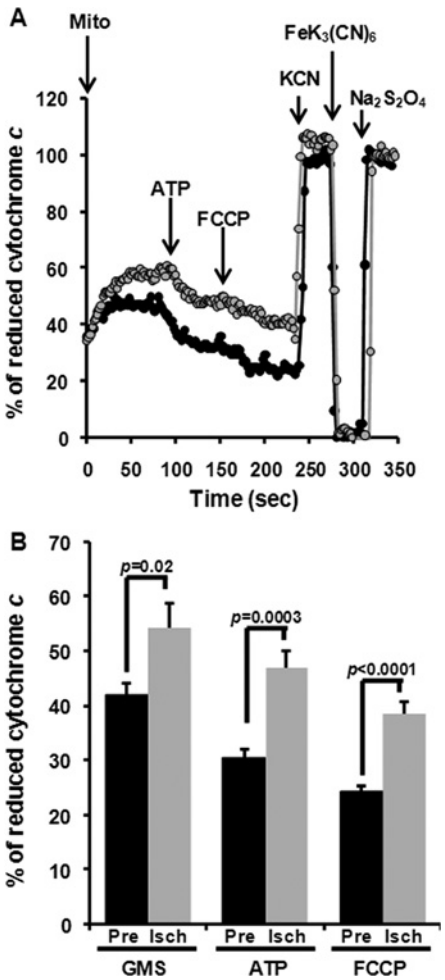

Figure 4 The effect of ischaemia on the redox state of cytochrome *c*

Mitochondria were incubated in KCl buffer containing GMS plus 5 mM creatine and creatine kinase (160 μg/mg protein). (**A**) 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 $FeK_3(CN)_6$ or 7 mM $Na_2S_2O_4$. Results were normalized to fully oxidized ($FeK_3(CN)_6$) and fully reduced ($Na_2S_2O_4$) values. (**B**) Mean data ($\pm$ S.E.M.; $n = 5$) for the percentage 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.

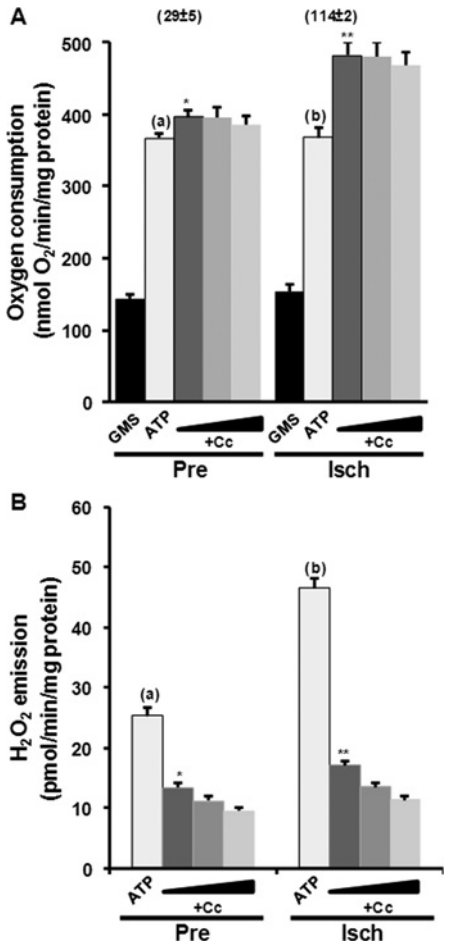

Figure 5 The effect of ischaemia on rates of oxygen consumption and H_2O_2 production in State 3.5

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. (**A**) Results for the oxygen consumption of pre-ischaemic (Pre) and end-ischaemic (Isch) mitochondria monitored before (black bars) and after addition of 200 μM ATP in the absence (white bars) or presence of 10, 25 or 50 μM cytochrome *c* (Cc; dark grey, medium grey and light grey bars respectively). 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*. (**B**) Results for the rate of H_2O_2 production for the same groups monitored after the addition of 200 μM ATP in the absence (white bars) or presence of 10, 25 and 50 μM cytochrome *c* (Cc; dark grey, medium grey and light grey bars respectively). All results are means $\pm$ S.E.M. of five separate mitochondrial preparations for the two groups. (**A**) $^*P < 0.05$ compared with (a), $^{**}P < 0.0001$ compared with (b). (**B**) $^*P < 0.0001$ compared with (a), $^{**}P < 0.0001$ compared with (b).

of KCN, end-ischaemic mitochondria showed a significantly lower NAD(P)H redox state that 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 fall in ROS production and further increase in NAD(P)H fluorescence. In Figure 7, we re-plot the results for the content of oxidized cytochrome *c* at low [KCN] (0–10 μM) against the rates of oxygen consumption (Figure 7A), H_2O_2 emission (Figure 7B) and NAD(P)H autofluorescence (Figure 7C). 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_2O_2 emission the results appear to fall on the same line for both sets of mitochondria. This is consistent with the greater H_2O_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_2O_2 emission (Figure 7C).

Cytochrome *c* loss from end-ischaemic mitochondria is associated with HK2 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

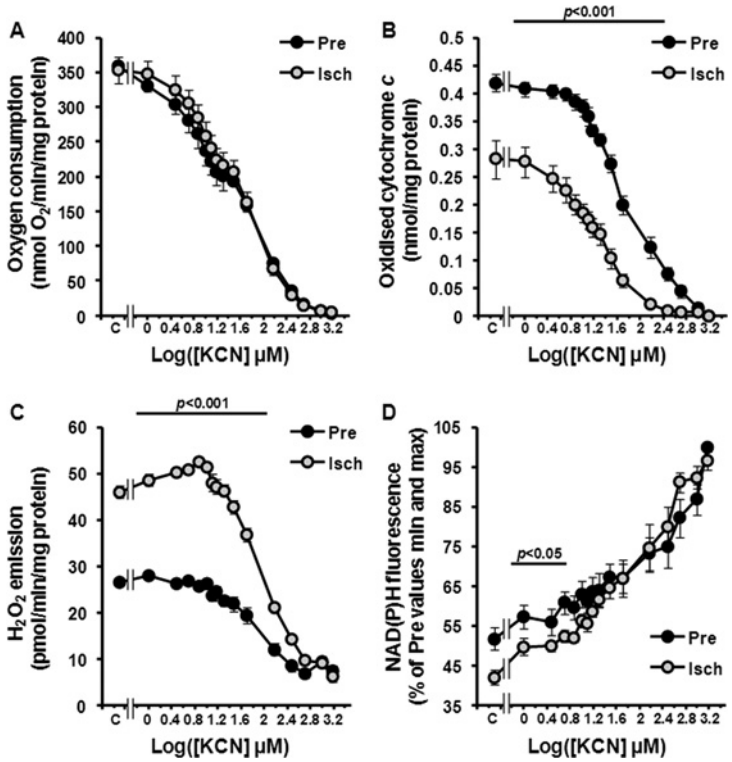

Figure 6 The effects of increasing [KCN] on mitochondrial function

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). (**A**) Mean data ($\pm$S.E.M.; $n = 5$) for the oxygen consumption of pre-ischaemic (Pre, black circles) and end-ischaemic (Isch, grey circles) mitochondria. (**B**–**D**) Parallel data for oxidized cytochrome *c*, H_2O_2 production and NAD(P)H autofluorescence respectively. For NAD(P)H fluorescence (**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 $\pm$ 0.4 and 35.9 $\pm$ 0.8 ($P = 0.63$) for pre-ischaemic and end-ischaemic mitochondria respectively. Results for the two groups are presented as means $\pm$ S.E.M. for five separate mitochondrial preparations with bars indicating *P* values Isch compared with Pre.

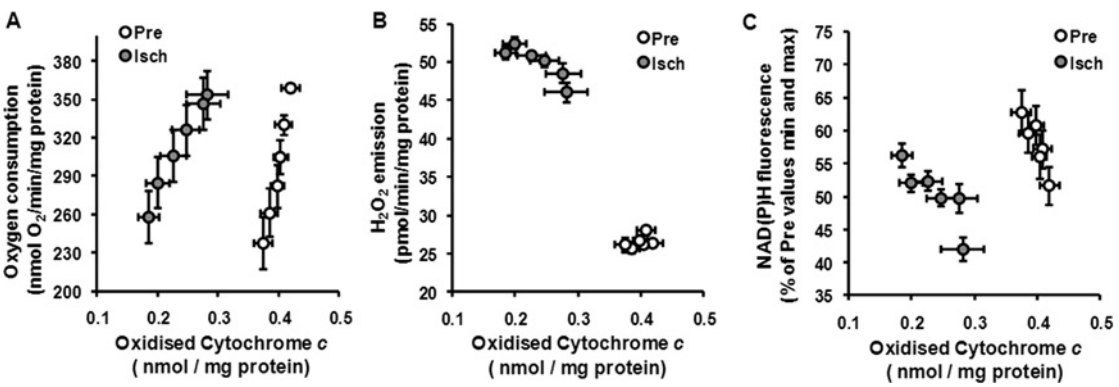

Figure 7 The relationship between H_2O_2 emission, oxygen consumption, NAD(P)H fluorescence and the content of oxidized cytochrome *c*

Pre-ischaemic (white circles) and end-ischaemic (grey circles) results obtained at low [KCN] (0–1 μM) for oxygen consumption (**A**), H_2O_2 emission (**B**) and NAD(P)H autofluorescence (**C**) were taken from Figure 6 and replotted as a function of the total content of oxidized cytochrome *c*.

mitochondria showed no change in the ratio Bid/t-Bid or P-Bad/Bad (Supplementary Figures S3A and S3B available at http://www.BiochemJ.org/bj/436/bj4360493add.htm). Changes in Bax or Bak were not detected (Supplementary Figures S3C and S3D). However, total 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 results of the present study 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 IP (ischaemic pre-conditioning) may also provide an explanation for the

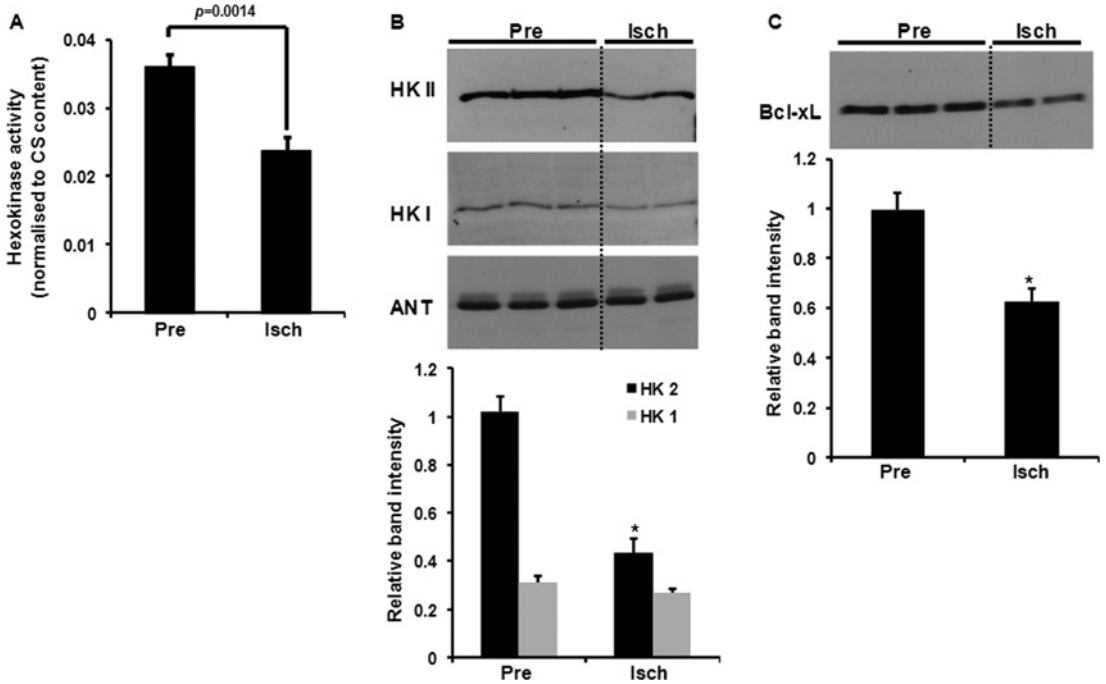

Figure 8 Effects of ischaemia on the mitochondrial content of HK and Bcl-xL

(**A**) HK-specific activity (means ± S.E.M.; $n = 6$) normalized to citrate synthase content as described in the Materials and methods section. HK-specific activity was 58.7 ± 2.7 and 37.3 ± 3.8 nmol · min^{-1} · mg protein^{-1} in Pre and Isch mitochondria respectively. (**B**) Top panel: typical Western blot used to determine the mitochondrial content of HK1, HK2 and ANT. 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 compared with Pre HK 2. (**C**) Top panel: shows a typical Western blot used to determine the mitochondrial content of Bcl-xL. 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 compared with Pre Bcl-xL.

mitochondrial production of ROS during IP that play a key signalling 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)physiologicial 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 favour 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 H_2O_2 production (Supplementary Figure S2A) in agreement with the results of others (see [2] for review). We did not observe any differences in H_2O_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) (results not shown). 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 the present study, mitochondrial respiration was stimulated by an ADP-regenerating system to produce an NADH:NAD$^+$ ratio and protonmotive force (Δp) that are relatively low, and rate of respiration quite high compared with State 4 conditions. Under these more physiological conditions, we did observe an increase in H_2O_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 H_2O_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 H_2O_2 production

Oxidized cytochrome *c* can scavenge superoxide in the IMS without its conversion into H_2O_2 that occurs spontaneously, but is enhanced by SOD. Scavenging superoxide independently of H_2O_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 H_2O_2 production and hydroxyl radical formation and depletion of GSH, all of which have been observed following ischaemia and reperfusion [27]. Our results 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 H_2O_2 production (Figure 5B). In addition, cytochrome *c* loss will

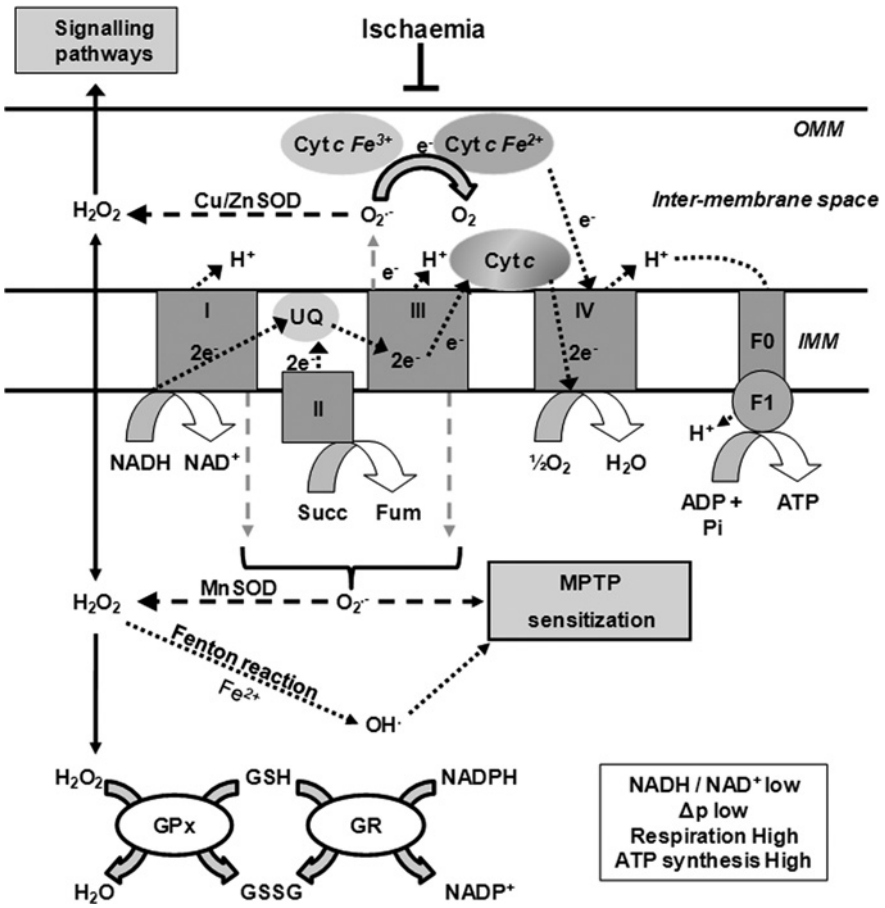

Figure 9 Scheme illustrating the role of oxidized cytochrome *c* in mitochondrial ROS production

Mitochondria are shown under conditions in which they are making ATP (State 3.5) and thus are characterized by a lower protonmotive force and NADH:NAD^+ ratio and higher rate of oxygen consumption than in State 4. Ischaemia decreases the available oxidized cytochrome *c* (Cyt *c*) in the IMS, thus impairing its scavenging ability and increasing the net rate of H_2O_2 production by mitochondria. The increased H_2O_2 can diffuse into the matrix thus challenging the detoxification system (GPx, glutathione peroxidase; GR, glutathione reductase) 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 broken transport chain that could be responsible for superoxide production (grey broken 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 IMS and formation of H_2O_2 that activates signalling pathways. Fum, fumarate; Succ, succinate; UQ, ubiquinone.

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 results 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 GSH observed during ischaemia [27,28]. Within the matrix detoxification of H_2O_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 H_2O_2 that can enter the matrix and oxidize the GSH pool leading to depletion of NADPH. This conclusion is further supported by the demonstration that short exposure of mitochondria to H_2O_2 leads to greater subsequent H_2O_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 results 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 CsA or sanglifehrin 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 IP, post-conditioning and TP (temperature pre-conditioning) 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].

NO 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 DEA (diethylamine NONOate diethylammonium salt) at concentrations between 2.5 and 10 μM. These concentrations of DEA caused an inhibition of State 3.5 respiration similar to those cyanide concentrations that induced increased H_2O_2 production in ischaemic mitochondria (Supplementary Figure S5A available at http://www.BiochemJ.org/bj/436/bj4360493add.htm). However, unlike cyanide the presence of DEA decreased rather than increased H_2O_2 production under all conditions tested (Supplementary Figure S5B). Since NO rapidly reacts with superoxide to produce peroxynitrite [40], it is likely that the decrease in H_2O_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_2O_2 by purified xanthine oxidase in the presence of SOD (Supplementary Figure S6 available at http://www.BiochemJ.org/bj/436/bj4360493add.htm). 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). Our results do not 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 (results not shown) were both without effect. These results differ from those of Borutaite et al. [15], who observed similar cytochrome *c* loss but found it to be CsA-sensitive. We cannot explain this discrepancy, but our results 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 results 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 pro-caspase 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 min of ischaemia (Supplementary Figure S4A available at http://www.BiochemJ.org/bj/436/bj4360493add.htm). We did detect increased cleavage of the DEVD peptide in cytosolic extracts of ischaemic hearts when compared with 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 S4B). 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. [48] reported that although ischaemia induced m-calpain translocation to the membrane, this was not associated with an increase in its basal activity.

A role for HK2 and Bcl-xL

It has been proposed that HK2 binding to mitochondria plays an anti-apoptotic role and inhibits mPTP opening [17,49]. HK2 is overexpressed 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 results 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 VDAC (voltage-dependent anion channel) and evidence has been presented for the regulation of binding through VDAC1 phosphorylation [17]. However, studies with mitochondria from VDAC1 knockout mice cast doubt on this [49]. Pro-survival signalling pathways such as those mediated by Akt (protein kinase B) phosphorylation and inhibition of GSK3β (glycogen synthase kinase 3β) 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 signalling 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 OMMs 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 Bcl-2 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 (Figure 8). In this context, it should be noted that Bcl-xL overexpression 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 signalling

Only the oxidized form of cytochrome *c* scavenges superoxide and thus any condition that produces an increase in cytochrome *c* reduction state without greatly depolarizing mitochondria would be expected to enhance superoxide production in the IMS and subsequently H_2O_2 levels in the cytosol. Our results employing low concentrations of cyanide to give a modest increase in cytochrome *c* reduction state confirm this (Figures 7B and 7C). 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 IMS consistent with this proposal [61]. There is also evidence that in hypoxia the regulation of gene expression through stabilization of HIF-1α (hypoxia induced factor-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.

AUTHOR CONTRIBUTION

Philippe Pasdois and Andrew Halestrap devised and supervised the project with input from Elinor Griffiths. All the experiments were designed and performed by Philippe Pasdois, except for the Western blot studies which were carried out by Joanne Parker The paper was written by Philippe Pasdois and Andrew Halestrap.

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online data

SUPPLEMENTARY ONLINE DATA

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

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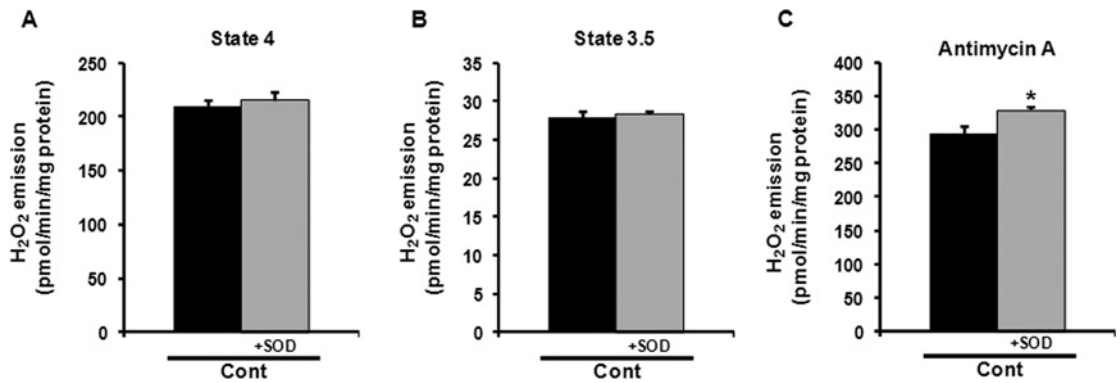

Figure S1 The effect of exogenous superoxide dismutase (SOD) on mitochondrial H_2O_2 production

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 of the main paper. (**A**) Rate of H_2O_2 production determined in State 4 in the absence (black bar) or presence of 5 units/ml SOD (grey bar). (**B**) Rate of H_2O_2 production measured in State 3.5 (addition of 200 μM ATP) in the absence (black bar) or presence of 5 units/ml of SOD (grey bar). (**C**) Rate of H_2O_2 production determined in the presence of antimycin A (2 μg/mg mitochondrial protein) in the absence (black bar) or presence of 5 units/ml of SOD. All results are means $\pm$ S.E.M. of three separate mitochondrial preparations. (**C**) $^*P < 0.05$ compared with without SOD.

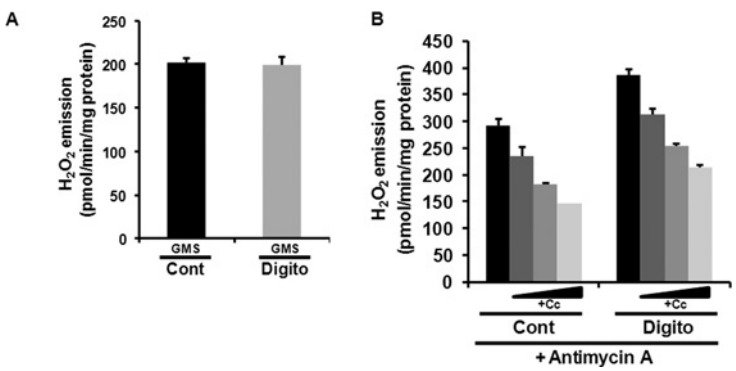

Figure S2 The effects of cytochrome *c* depletion on mitochondrial H_2O_2 production by mitochondria in State 4 and in the presence of antimycin A

Control mitochondria (Cont) were isolated according to the protocol described under Materials and methods section of the main paper. (**A**) Mitochondrial H_2O_2 production was monitored in State 4 after the addition of GMS. In a parallel group, energized mitochondria were treated with digitonin (Digito, grey bars). (**B**) Control (Cont) or digitonin-treated (Digito) mitochondria were incubated with antimycin A (2 μg/mg mitochondria) in the absence (solid bars) or presence of three different concentrations of cytochrome *c* (10, 25 or 50 μM, dark grey, medium grey and light grey bars respectively).

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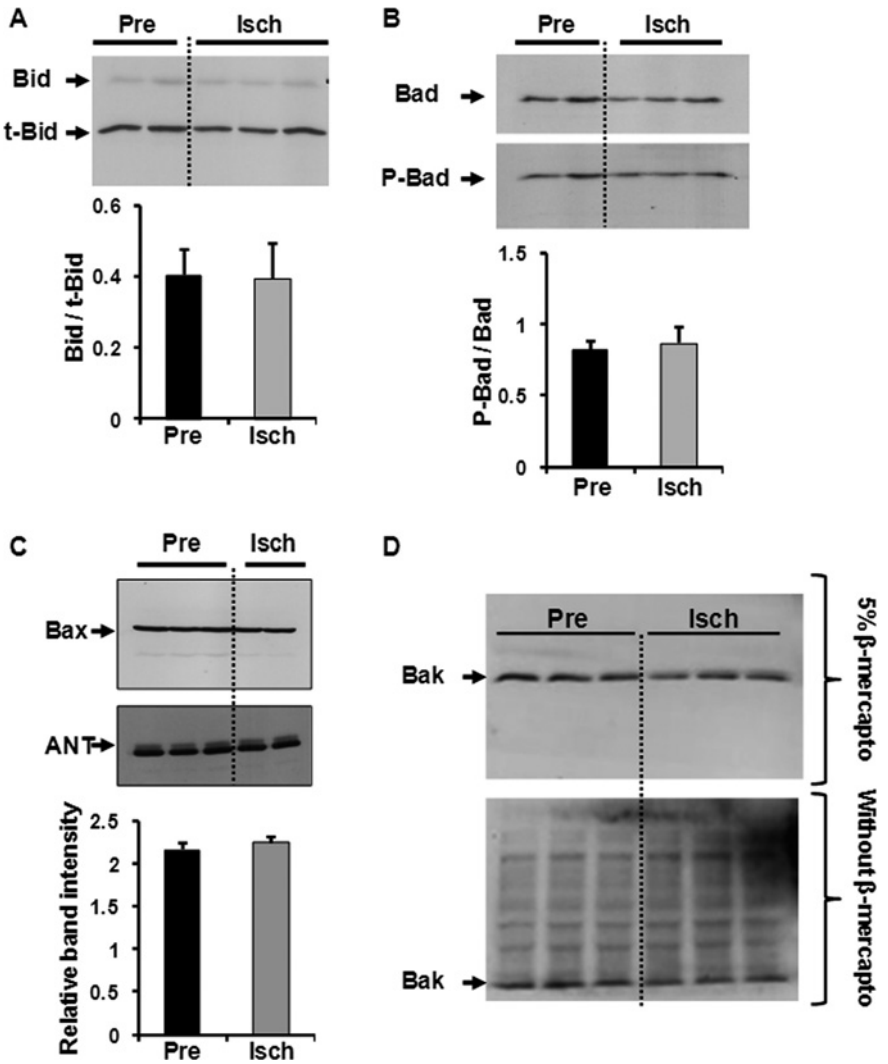

Figure S3 Ischaemia did not change the content of Bid, Bad, Bax or Bak

Mitochondrial fractions were subject to SDS/PAGE, followed by Western blotting with the appropriate antibody for the detection of Bid, t-Bid (**A**), Bad, P-Bad (**B**), Bax (**C**) or Bak (**D**) as described in the Materials and methods section of the main paper. (**A** and **B**) Representative blots and mean data ($\pm$ S.E.M., $n = 5$) of the mitochondrial ratio of Bid to t-Bid and P-Bad/Bad respectively. (**C**) Representative blots and mean data ($\pm$ S.E.M., $n = 5$) of the mitochondrial ratio of Bax/ANT (adenine nucleotide translocator). (**D**) Representative blots in the presence (top) or absence (bottom) of 5 % 2-mercaptoethanol (β-mercapto), the latter allowing detection of changes in oligomeric Bak [1]. Isch, ischaemia; Pre, pre-ischaemia.

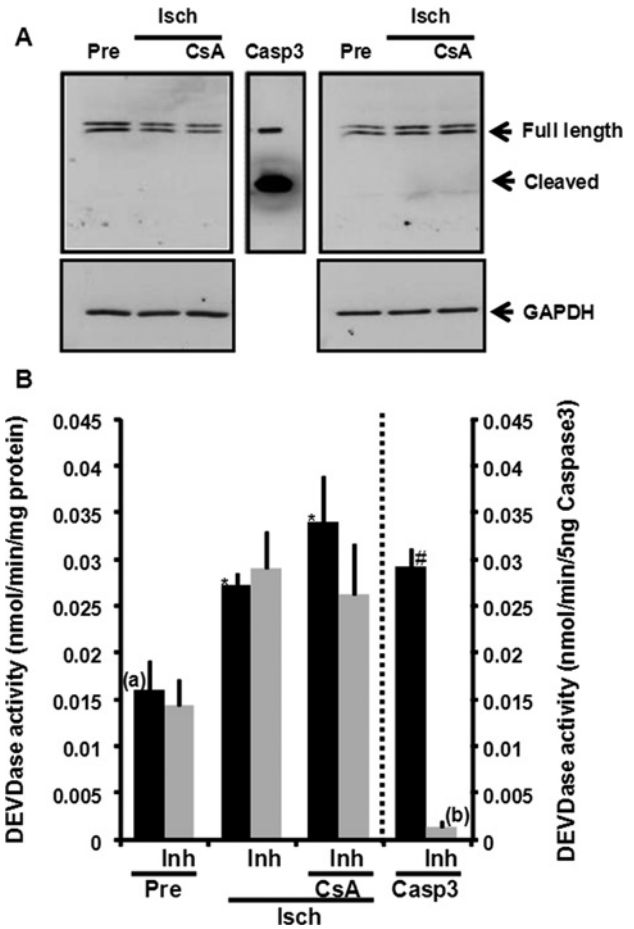

Figure S4 The effect of ischaemia on caspase 3 cleavage and DEVDase activity

Hearts were freeze-clamped before ischaemia (Pre) or after 30 min ischaemia with or without 0.2 μM cyclosporine A (Isch, Isch + CsA). Cytosolic fractions were prepared according to the protocol described under Materials and methods section of the main paper. (**A**) Representative Western blot of two independent cytosolic fractions for each group, and for a full-length and cleaved recombinant caspase 3. (**B**) Rate at which the caspase 3 substrate (DEVD peptide) was cleaved *in vitro* in the absence (black bars) or presence (grey bars) of the caspase 3 inhibitor DEVD-CHO (Inh). Results are means $\pm$ S.E.M. of four (Isch + CsA) or six independent cytosolic fractions for each group. $*P < 0.02$ compared with (a), $\#P < 0.0001$ compared with (b).

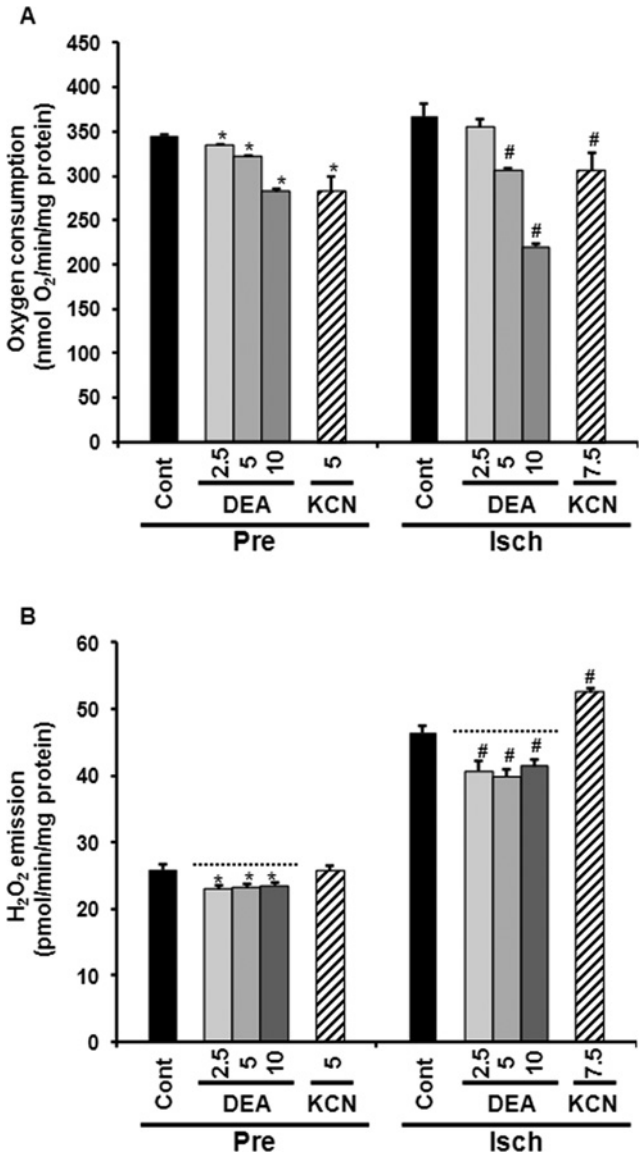

Figure S5 The effect of DEA on oxygen consumption and H_2O_2 emission

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 of the main paper. (**A**) Results for the oxygen consumption of pre-ischaemic (Pre) and end-ischaemic (Isch) mitochondria monitored after addition of 200 μM ATP in the absence (black bars) or presence (gradient grey bars) of the NO-donor DEA or the presence (hatched bars) of potassium cyanide (KCN). (**B**) Results for the rate of H_2O_2 production for the same groups monitored after the addition of 200 μM ATP in the absence (black bars) or presence (gradient grey bars) of DEA or presence (hatched bars) of KCN. For DEA treatment, oxygen consumption and H_2O_2 emission were monitored for 3 min after DEA addition and each bar represents the average effect obtained over this period of time. All results are means $\pm$ S.E.M. of three separate mitochondrial preparations for the two groups. $*P < 0.05$ compared with (Cont Pre), $\#P < 0.05$ compared with (Cont Isch).

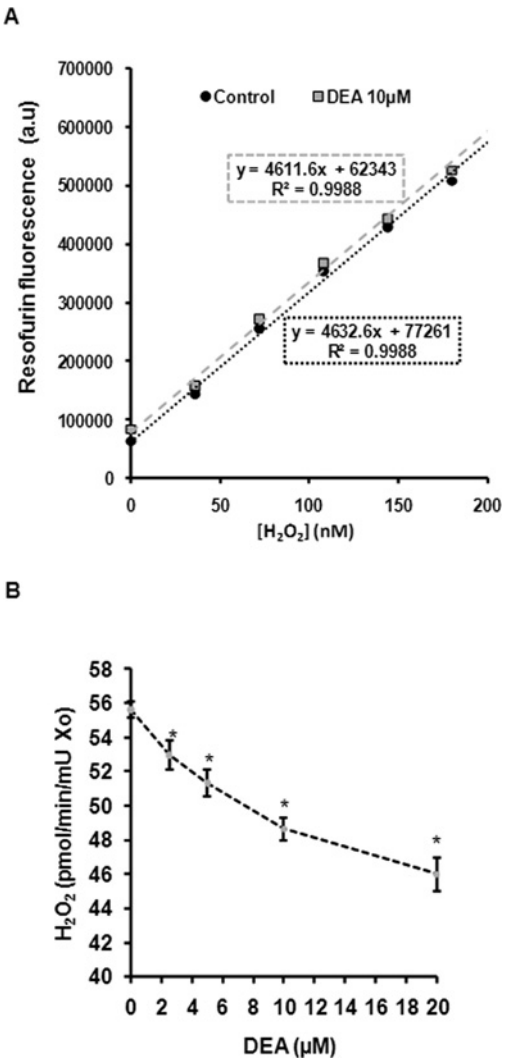

Figure S6 The effect of DEA on superoxide production by xanthine oxidase in the presence of SOD

(**A**) Calibration curves for Amplex Red detection of H_2O_2 performed in the absence (black line) or presence (grey line) of 10 μM DEA. Measurements were performed at 37 °C in a KCl buffer containing 30 μM Amplex Red and 0.1 mg/ml peroxidase. (**B**) Results for the effect of increasing concentrations of DEA on the rate of H_2O_2 production generated by the mix xanthine (110 μM) and xanthine oxidase (0.1 unit/ml) in the presence of SOD (10 units/ml). *$P < 0.02$ compared with (without DEA).

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