Inhibition of Mitochondrial Permeability Transition Pore Opening by Ischemic Preconditioning Is Probably Mediated by Reduction of Oxidative Stress Rather Than Mitochondrial Protein Phosphorylation

Samantha J. Clarke,* Igor Khaliulin,* Manika Das, Joanne E. Parker, Kate J. Heesom, Andrew P. Halestrap

Abstract—Inhibition of mitochondrial permeability transition pore (MPTP) opening at reperfusion is critical for cardioprotection by ischemic preconditioning (IP). Some studies have implicated mitochondrial protein phosphorylation in this effect. Here we confirm that mitochondria rapidly isolated from preischemic control and IP hearts show no significant difference in calcium-mediated MPTP opening, whereas IP inhibits MPTP opening in mitochondria isolated from IP hearts following 30 minutes of global normothermic ischemia or 3 minutes of reperfusion. Analysis of protein phosphorylation in density-gradient purified mitochondria was performed using both 2D and 1D electrophoresis, with detection of phosphoproteins using Pro-Q Diamond or phospho-amino-specific antibodies. Several phosphoproteins were detected, including voltage-dependent anion channels isoforms 1 and 2, but none showed significant IP-mediated changes either before ischemia or during ischemia and reperfusion, and neither Western blotting nor 2D fluorescence difference gel electrophoresis detected translocation of protein kinase C (α, ε, or δ isoforms), glycogen synthase kinase 3β, or Akt to the mitochondria following IP. In freeze-clamped hearts, changes in phosphorylation of GSK3β, Akt, and AMP-activated protein kinase were detected following ischemia and reperfusion but no IP-mediated changes correlated with MPTP inhibition or cardioprotection. However, measurement of mitochondrial protein carbonylation, a surrogate marker for oxidative stress, suggested that a reduction in mitochondrial oxidative stress at the end of ischemia and during reperfusion may account for IP-mediated inhibition of MPTP. The signaling pathways mediating this effect and maintaining it during reperfusion are discussed. (Circ Res. 2008;102:1082-1090.)

Key Words: mitochondrial permeability transition ▪ preconditioning ▪ reperfusion injury ▪ protein phosphorylation ▪ oxidative stress

A critical factor mediating reperfusion injury of the heart is the mitochondrial permeability transition pore (MPTP), the opening of which causes mitochondrial swelling with release of proapoptotic proteins and uncoupling of mitochondrial oxidative phosphorylation. The resulting ATP deprivation causes disruption of ionic homeostasis and contractile function and ultimately sarcolemma rupture and necrosis. Inhibition of MPTP opening during reperfusion protects hearts from reperfusion injury, and effective cardioprotection is also mediated by ischemic preconditioning (IP) before prolonged ischemia is initiated, and this also involves inhibition of MPTP opening.

Extensive evidence points to protein kinase (PK)C playing a central role in IP, although controversy remains over which PKC isoform(s) are involved and their translocation to mitochondria. The strongest evidence implicates PKCε because PKCε-knockout mice do not exhibit IP and transgenic mice with cardiac-specific overexpression of PKCε or expression of a PKCε activator are protected from reperfusion injury. Several studies have reported PKCε translocation to the particulate fraction, including mitochondria, where it may phosphorylate putative components of the MPTP such as the voltage-dependent anion channel (VDAC). Others have proposed that activation of cyclic-GMP–dependent PK (PKG) by nitric oxide activates a mitochondrial intermembrane pool of PKCε, leading to opening of mitochondrial ATP-sensitive potassium channels, followed by reactive oxygen species (ROS) formation, activation of a distinct mitochondrial PKCε pool, and, finally, inhibition of the MPTP. Activation of prosurvival kinases such as Akt, especially during reperfusion, has been implicated by others, whereas Sollott and colleagues have proposed...
that protection by all these kinase may converge to phosphoryl-
ate and inhibit glycogen synthase kinase (GSK)3β. However, no mitochondrial phosphoprotein has been identified that may
mediate protection by these kinases.

In this report, we investigate which PKs associate with
carefully purified mitochondria from control and IP hearts
and analyze whether consistent changes in mitochondrial
protein phosphorylation can be detected. Neither approach
provided evidence for IP mediating its effects by mitochon-
drial protein phosphorylation, but we confirm that mitochon-
drial protein oxidation is decreased by IP at the end of
ischemia and early reperfusion. We propose that this reduc-
tion in oxidative stress experienced by mitochondria, rather
than protein phosphorylation, may be responsible for IP-
mediated inhibition of MPTP at the start of reperfusion.

Materials and Methods

Materials

The sources of all materials are described in online data supplement,

Heart Perfusion

All procedures conformed to the UK Animals (Scientific Procedures)
Act 1986 and the Guide for the Care and Use of Laboratory Animals
published by the National Institutes of Health (NIH Publication No.
85-23, revised 1996). Langendorff perfusions of hearts from male
Wistar rats (250 to 260 g) were performed as described previously16
and are detailed in the expanded Methods section in the online data
supplement. All hearts experienced 35 minutes of preischemia,
which included the required treatment shown schematically in Figure
1. Perfusate was sampled for determining lactate dehydrogenase
(LDH) activity. Table I in the online data supplement presents data
on hemodynamic function and LDH release, confirming cardiopro-
tection by IP similar to that observed previously.3-5,17,18 At the
required time (Figure 1), hearts were either rapidly homogenized for
the preparation of mitochondria or freeze-clamped using liquid
nitrogen-cooled tongues, ground under liquid nitrogen, and stored at
−80°C for later analysis.

Isolation and Analysis of Particulate and
Mitochondrial Fractions

All procedures were carried out at 0°C to 4°C in buffers containing
protease and phosphatase inhibitors. Two protocols were used to
prepare mitochondrial and particulate fractions from homogenized
hearts or frozen heart powder as detailed in the expanded Methods
section.

PKC Translocation and Protein Phosphorylation

Fractions (10 to 25 μg protein) were analyzed by SDS-PAGE and
Western blotting with antibodies against both specific phosphopro-
teins and the corresponding total protein and then quantification by
scanning (see the expanded Methods section). The ratio of the band
intensity for phosphoprotein to total protein was used as a measure
of phosphorylation state. Purity of mitochondrial fractions was
assessed by Western blotting with antibodies against the adenine
nucleotide translocase and monocarboxylate transporter 1, a specific
plasma membrane marker.19

Two-Dimensional Gel Electrophoresis and
Two-Dimensional Difference Gel Electrophoresis

These were performed in the University of Bristol Proteomics
Facility as described in the expanded Methods section, and gels were
visualizedfluorescently(differegencegel electrophoresis)orstained
for phosphoproteins and total protein (Pro-Q Diamond and Sypro–
Ruby, Invitrogen).

Measurement of MPTP Opening In Vitro and
Protein Carbonylation Assays

MPTP opening was determined at 25°C under deenergized condi-
tions by monitoring A520 whereas protein carbonyls were analyzed
by derivatization with dinitrophenylhydrazine, followed by Western
blotting,5,18 as detailed in the expanded Methods section.

Figure 2. MPTP opening and protein carbonylation in mitochon-
dria from control and IP hearts. A shows the maximum
decline in A520 after calcium addition to deenergized mitochon-
dria. B shows a representative western blot for protein car-
boxylase (CP) (nonischemic control hearts). Data in A and C
are presented as means of 6 or 12 (preischemic) separate mito-
chondrial preparations from each group (IP vs control: *P<0.05,
**P<0.01).

Figure 1. Protocols used for heart perfusion. The times at which
control (C) and IP hearts were freeze clamped or homogenized
for mitochondrial preparation are indicated by arrows. For preis-
chemic IP heart samples were taken at the 2 points indicated as
IP# and IP.
Results

MPTP Opening Was Inhibited in Mitochondria Isolated From IP Hearts Only After Ischemia

Figure 2A confirms that mitochondria isolated from IP hearts at the end of ischemia or after 3 minutes of reperfusion exhibited less calcium-induced MPT opening than those isolated from control hearts, whereas no differences were observed in mitochondria isolated immediately after the IP protocol.3-5 This lack of effect of IP was independent of the [Ca^{2+}] used (supplemental Figure I). Because our protocol uses deenergized mitochondria with buffered [Ca^{2+}] in the presence of calcium-ionophore (A23187), the IP-mediated inhibition of MPTP opening cannot be caused by either differences in mitochondrial calcium loading or membrane potential. Rather, it must reflect a change in calcium sensitivity of the MPTP. Two possible mechanisms may account for this: either phosphorylation of some MPTP component or oxidative modification of critical thiol groups on the MPTP that sensitize it to [Ca^{2+}].1

Mitochondrial Protein Oxidation Was Decreased by Preconditioning

The data of Figure 2B and 2C confirm that mitochondria isolated from IP hearts during reperfusion exhibit less protein carbonylation, a measure of protein oxidation and a surrogate marker of mitochondrial oxidative stress,5 whereas no effect of IP was observed in preischemic hearts. Previous work has shown a similar reduction in protein carbonylation by IP and other preconditioning stimuli at the end of ischemia5 and with temperature preconditioning, urocortin, and apomorphine at reperfusion.18,20,21

Translocation of Protein Kinases to Purified Mitochondria Was Not Detected Following IP

Activation of PKC isoforms causes their translocation to intracellular membranes, including the plasma membrane.22 Because conventionally prepared mitochondria are contaminated with such membranes,23 their complete removal is essential when studying PKC translocation to mitochondria. Two protocols were used to achieve this, as illustrated in supplemental Figure I. Either a cytosolic fraction and crude high-speed particulate fraction were prepared, followed by preparation of mitochondria from the latter (protocol 1) or a more conventional mitochondrial preparation was used (protocol 2). In both cases, contaminating plasma membranes were removed by Percoll gradient centrifugation. Figure 3 shows that protocol 1 produced a crude particulate fraction containing both mitochondria (adenine nucleotide translocase) and plasma membranes (monocarboxylate transporter 1), as did the crude mitochondrial preparation of protocol 2 (Figure 4). In both cases, Percoll purification removed plasma membranes (monocarboxylate transporter 1), with the loss of almost all the PKCα and PKCe. Although PKCδ remained, no increase was detected following IP treatment, whether mitochondria were isolated before ischemia (Figures 3A and 4A) or during reperfusion (Figure 3C). Treatment with 50 μmol/L diazoxide, an IP mimic, did not cause detectable PKCα or PKCe translocation, whereas treatment with phorbol ester (200 μmol/L phorbol ester (phorbol-12-myristate-13-acetate [TPA])) produced the anticipated loss of both isoforms from the cytosol and a slight increase in the crude particulate fraction (Figure 3A). A larger PKC increase was detected in the plasma membrane fraction (Figure 4A), ensuring a robust response in the particulate:cytosolic ratio

![Figure 3](image-url)

Figure 3. IP does not cause translocation of PKC isoforms to mitochondria. Subcellular fractionation of hearts into cytosolic (Cyt), crude mitochondria (Crd), and pure mitochondria (Mit) was performed according to protocol 1 (supplemental Figure I). A and B represent fractions isolated immediately after IP or following 10 minutes of treatment with 50 μmol/L diazoxide or 0.2 μmol/L phorbol ester, whereas C and D represent fractions isolated after 30 minutes ischemia and 3 minutes of reperfusion. Proteins were separated by SDS-PAGE, followed by Western blotting with the appropriate antibody. B and D present mean data (n=6) of the ratio of mitochondrial or crude particulate PKC to cytosolic PKC.
Determination of AMPK, GSK3β, and Akt phosphorylation in Freeze-Clamped Hearts

We investigated the phosphorylation state of AMPK, GSK3β, and Akt in freeze-clamped control and IP hearts. Extracts were rapidly prepared in the presence of phosphatase and protease inhibitors, and proteins were separated by SDS-PAGE before Western blotting with antibodies against the phosphorylated and total kinases. Representative blots and mean data for the ratio of phosphorylated to total protein are shown in Figure 5. Before index ischemia, samples from IP hearts were taken either at the end of the third brief ischemic phase of preconditioning or immediately before ischemia, as indicated in Figure 1. In A, where 2 adjacent control samples (C) are shown, they represent 2 separate hearts. *P<0.05, **P<0.02, ***P<0.01 vs preischemic control.

**Figure 5.** Cytosolic AMPK, Akt, GSK3β, and ACC phosphorylation state in freeze-clamped hearts. Control and IP hearts were freeze-clamped at the times shown in Figure 1, and a cytosolic fraction was produced according to the freeze-clamp protocol of supplemental Figure I. Proteins were separated by SDS-PAGE, followed by Western blotting with the appropriate antibody for the total (t) or phosphorylated (p) kinases indicated or for ACC. A shows representative blots, whereas B shows mean data (n=6) of scanned blots where the ratio of phosphorylated to total protein is expressed relative to the ratio for the control preischemic sample run on the same gel. IP# and IP samples represent samples taken from IP hearts at the end of the third brief ischemic phase of preconditioning or immediately before ischemia, as indicated in Figure 1. In A, where 2 adjacent control samples (C) are shown, they represent 2 separate hearts. *P<0.05, **P<0.02, ***P<0.01 vs preischemic control.
before ischemia. Samples taken after 5 minutes of index ischemia showed a substantial increase in AMPK phosphorylation relative to preischemic values and small increases in the phosphorylation of ACC, GSK3β, and Akt, but in no case could we detect a significant difference between control and IP hearts. After 30 minutes of ischemia, AMPK phosphorylation remained slightly elevated compared with the preischemic value. These data are consistent with previous observations that insulin must be present during reperfusion to be protective. Additional evidence against a role for AMPK in IP was provided by using compound C, an established inhibitor of AMPK, which exerted no effect on the ability of IP to improve hemodynamic function or lower LDH release (supplemental Table I) but does inhibit AMPK-mediated changes in ACC phosphorylation. By contrast, exposure to 10 μmol/L chelerythrine blocked the IP-mediated decrease in LDH release during reperfusion (supplemental Table I and Figure IV).

Preconditioning Gave No Detectable Changes in Mitochondrial Protein Phosphorylation

The phosphorylation state of proteins in mitochondria rapidly isolated in the presence of phosphatase and protease inhibitors was determined following their separation by 2D gel electrophoresis. Pro-Q Diamond and Sypro–Ruby were used to detect phosphoproteins and total proteins, respectively. Figure 7 presents phosphoprotein data for mitochondria from control and IP hearts isolated both before ischemia and after 30 minutes of ischemia and 3 minutes of reperfusion. In supplemental Figure VA, these data are overlaid (red) on the Sypro–Ruby protein data (green) to allow discrimination between truly phosphorylated proteins and proteins stained nonspecifically with the Pro-Q Diamond. Supplemental Figure VB shows similar data from another set of preischemic, end-ischemic, and reperfused hearts. A significant number of proteins were preferentially stained with the Pro-Q Diamond stain, implying that they were phosphorylated. By far the strongest signal was in the 40-kDa region (box 1) in the correct location for multiple phosphorylation states of the E1α subunit of pyruvate dehydrogenase (PDHE1α) (accession no. P26284, Swiss Prot; molecular mass, 40.2 kDa) that we have previously shown to be the dominant matrix phosphoprotein whose phosphorylation turns over rapidly. PDHE1α is known to exhibit multiple phosphorylation states, and the theoretical pI values of the 0, 1, 2, and 3 phosphorylated states of 6.82, 6.52, 6.31, and 6.15 are consistent with the spots observed. In supplemental Figure IX, we provide data to confirm the identity of these spots as PDH1Eα by using mass spectrometry (see supplemental Table II) and by Western blotting and dephosphorylation studies. Supplemental Figure IXs and Table II also provide data on the identity of the 2 phosphoprotein spots at ~31 kDa in box 2 of Figure 7. These were shown to be singly phosphorylated forms of the VDAC isoforms 1 and 2 (VDAC1, theoretical pI 7.83; VDAC2, theoretical pI 6.68). Evidence has been presented that VDAC1 can be phosphorylated and that this may lead, directly or indirectly, to inhibition of MPTP opening. However, our data revealed no changes in phosphorylation of either VDAC1 or VDAC2 in response to IP.

In supplemental Figure VI, we confirm that we were able to detect IP-mediated changes in cytosolic protein phosphorylation. As additional confirmation that IP exerted no effect on mitochondrial protein phosphorylation, we used 2D fluorescence difference gel electrophoresis in which mitochondrial proteins of control and IP hearts were labeled with red and green fluorescent probes before mixing and separating on the same 2D gel. Proteins unchanged by IP treatment run in the same place and show as yellow spots, whereas any differences are revealed as red or green spots. Data are shown in supplemental Figure VII and Table II for mitochondria from preischemic, end-ischemic, and reperfused hearts, but,
once again, no consistent IP-mediated changes were revealed that may account for MPTP inhibition.

We considered that IP may cause changes in the phosphorylation state of proteins, such as the adenine nucleotide translocase, that do not readily enter the isoelectric focusing gel and so would not be detected using the 2D gels. Thus, we also performed 1D SDS-PAGE with Pro-Q Diamond staining but, again, found no evidence for changes in any protein phosphorylation following IP (supplemental Figure VIII, A). We have also used antibodies against phosphotyrosine, phosphoserine, and phosphothreonine as an alternative strategy to detect IP-mediated changes in protein phosphorylation. Here too, no effects of IP were observed (supplemental Figure VIII, B through D).

**Discussion**

**Mitochondrial Protein Phosphorylation May Not Be Required for IP-Mediated Inhibition of MPTP Opening**

Mitochondria isolated immediately after IP showed no decrease in sensitivity to calcium-induced MPTP opening (Figure 2A and elsewhere3,5). These data argue against phosphorylation of a component of the MPTP during the IP protocol mediating inhibition of pore opening, and our inability to detect a change in mitochondrial protein phosphorylation at this time (Figure 7 and supplemental Figures V, VII, and IX) support this conclusion. However, we were also unable to detect an IP-induced change in the phosphorylation state of any mitochondrial protein in IP hearts at the end of ischemia or during reperfusion (Figure 7 and supplemental Figures V and VII through IX), at which time MPTP opening was inhibited by IP (Figure 2A and elsewhere4,5). Thus, it seems unlikely that inhibition of MPTP opening at reperfusion is mediated by protein phosphorylation. Our inability to detect translocation of AMPK, Akt, GSK3β, or PKC isoforms to the mitochondria (Figures 3 and 4) or changes in their phosphorylation state (Figure 4 and supplemental Figures VII and VIII) is consistent with this conclusion. Although several groups have reported that IP causes translocation of PKC isoforms, particularly PKCe, to the particulate fraction,9,11,29–33 not all reported PKCe translocation to mitochondria.18,31,32 This may be because PKCe translocation is transient in the rat heart, being lost after 3 brief ischemic periods, as used in the present study.34 Some studies have reported IP-mediated PKCδ translocation to mitochondria.31,32 Although we confirmed the presence of PKCδ in the mitochondria we did not observe IP-mediated translocation (Figure 3).

We have not attempted to identify all the phosphoproteins detected in the mitochondria, but phosphorylated forms of...
PDHE1α were the dominant spots (Figure 7 and supplemental Figure IX). PDHE1α is the major phosphoprotein in the mitochondrial matrix known to be rapidly phosphorylated and dephosphorylated, together with a small amount of the E1α subunit of branch-chain keto-acid dehydrogenase (BCKDH E1α).25 The additional phosphoproteins detected by us and others24 may turn over more slowly and so are not detected using rapid labeling with 32P. Alternatively, they may represent proteins integral to the outer mitochondrial membrane such as VDAC1 and VDAC2 (supplemental Figure IX and elsewhere27) or bound to it using scaffolding proteins.35,36 Their phosphorylation could be regulated by cytosolic kinases associating weakly with mitochondria but not remaining bound during isolation. Indeed, we have shown previously that when mitochondria are isolated from hepatocytes incubated with 32Pi, additional phosphoproteins are observed with 2 proteins of 30 to 35 kDa, demonstrating increased phosphorylation following glucagon treatment.37,38 Whatever their identity, it seems unlikely that the phosphoproteins we detect are involved in IP-mediated inhibition of MPTP opening because their phosphorylation is not changed by IP. This includes VDAC1, the phosphorylation of which has been proposed by others to regulate the MPTP.11,28

We cannot totally rule out a role for protein phosphorylation regulating the MPTP because there may be phosphoproteins present at levels below the detection limit of Pro-Q Diamond, difference gel electrophoresis, or phospho–amino acid–specific antibodies. Additionally, dephosphorylation may have occurred during the mitochondrial preparation despite the presence of phosphatase inhibitors, although this is unlikely because we detected many phosphoproteins in both mitochondrial and cytosolic fractions with IP-mediated changes in the latter (supplemental Figure VI).

Reduction in Oxidative Stress May Explain the Inhibition of MPTP Opening by IP
Preconditioning by a variety of means reduces oxidative stress following ischemia and reperfusion,39–43 and we have previously shown that this is associated with less oxidative damage to mitochondria as monitored by protein carbonylation.5,18,20,21 Here we confirm that this is the case for IP (Figure 2B and 2C). Because thiol oxidation greatly sensitizes the MPTP to [Ca2+]1, an IP-mediated decrease in oxidative damage provides sufficient explanation for the observed inhibition of MPTP opening at reperfusion, without the need to invoke mitochondrial protein phosphorylation. This would explain why there is no decrease in calcium sensitivity of MPTP opening immediately following IP, when there is no significant oxidative damage (Figure 2, supplemental Figure II, and elsewhere4). Such a mechanism is also consistent with the strong cardioprotection afforded by antioxidants specifically targeted to mitochondria.44 Thus, we propose that decreasing oxidative stress at the end of ischemia and during early reperfusion represents the common mechanism by which preconditioning stimuli inhibit MPTP opening.

The Signaling Pathways by Which Ischemic Preconditioning Reduces Oxidative Stress
Our data lead us to conclude that AMPK, Akt, or GSK3β are unlikely to be involved in reducing oxidative stress at the end of ischemia and early in reperfusion, as we found no appropriate changes in their phosphorylation state in response to IP (Figure 5) or diazoxide (supplemental Figure III). Nor did the AMPK inhibitor compound C prevent protection by IP as measured by either hemodynamic function or LDH release (supplemental Table I). Indeed, if anything, this reagent enhanced the hemodynamic recovery of IP hearts, which may reflect a metabolic effect of the inhibitor such as inhibition of fatty acid oxidation.18 By contrast, the inability to precondition PKCε-knockout mice,45 the ability of chelerythrine (supplemental Table I) and other PKC inhibitors to prevent IP, and the ability of PKC activation to mimic preconditioning7,29,46,47 all argue for a critical role of PKCε in this process. Furthermore, the decrease in oxidative stress at reperfusion caused by both urocortin21 and temperature preconditioning18 are mediated by PKC. However, the mechanism by which this is achieved and whether decreased ROS production or increased ROS removal is involved remains to be elucidated.

Additional Signaling Pathways May Inhibit MPTP Opening as Reperfusion Continues
Activation of prosurvival kinases such as members of the mitogen-activated protein kinase family and the phosphatidylinositol 3-kinase/Akt cascade during reperfusion have been proposed to play a key role in IP-mediated cardioprotection.48 Maintenance of the MPTP inhibition during reperfusion appears to be critical for cardioprotection because treatment with cyclosporin-A and sanglifehrin-A (MPTP inhibitors) within the first 15 minutes of reperfusion is sufficient to produce a profound reduction in infarct size.49,50 This may reflect an ongoing cascade of MPTP opening whereby the initial pore opening at reperfusion stimulates ROS production that goes on to cause further MPTP opening as reperfusion continues.51–52 This MPTP-mediated ROS production could involve cytochrome c release, caspase activation, and subsequent cleavage of the p75 subunit of complex I.53,54 Prevention of MPTP opening at the start of reperfusion, as occurs through IP reduction in ROS levels, will also prevent the subsequent MPTP opening and thus

![Figure 8](https://example.com/figure8.png)

Figure 8. Suggested pathways by which IP may lead to inhibition of MPTP opening during reperfusion. Active PKs are shaded gray. Further details are given in the text.
represents a protective mechanism with “memory,” as defined by Sollott and colleagues. By contrast, those stimuli, such as insulin, that affect the survival kinase pathway may act on the caspase-mediated pathway and so work only during reperfusion and thus lack “memory.” In Figure 8, we present a scheme that summarizes these proposals.

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**Disclosures**

None.

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Supplementary Material
Supplementary Materials and Methods

Antibodies and chemicals

Polyclonal antibodies were raised in rabbits against purified rat heart mitochondrial whole ANT and a C-terminal peptide of MCT1, conjugated to keyhole limpet hemocyanin, as described previously. The antibody against total acetyl CoA carboxylase (ACC) was a kind gift of Professor Dick Denton of this department. All other antibodies were purchased from the following sources: against PKC\(\alpha\), PKC\(\delta\) and PKC\(\varepsilon\) from Santa Cruz Biotechnology; against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Abcam; against total Akt, GSK3\(\beta\) and AMPK-\(\alpha\), and phosphorylated Akt (Ser473), GSK3\(\alpha/\beta\) (Ser21/9) and AMPK\(\alpha\), (Thr172) from Cell Signaling Technology; against phosphorylated ACC (Ser79) from Upstate; against phosphothreonine and phosphoserine from Qiagen; against PDHE1\(\alpha\) from MitoSciences, against dinitrophenyl from Sigma and against VDAC (all isoforms) from Calbiochem. Phorbol ester (phorbol-12-myristate-13-acetate), diazoxide and insulin were purchased from Sigma.

Heart Perfusion

All procedures conformed to the UK Animals (Scientific Procedures) Act 1986 and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Wistar rats (250-260 g) were killed by stunning and cervical dislocation and hearts (~0.75 g) 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\(^\circ\)C (pH 7.4). Langendorff heart perfusions were performed as described previously with measurement of hemodynamic function using a latex balloon in the left ventricle, inflated to give an end-diastolic pressure of 2.5-5 mm Hg. Hemodynamic data were analyzed using a Data Acquisition System (PowerLab System, ADInstruments, Australia). Left ventricular
developed pressure (LVDP) was calculated as the difference between left ventricular systolic (LVSP) and end-diastolic pressures (LVEDP), and work index (RPP) as the product of LVDP and heart rate (HR).

**Experimental Protocols** All hearts were subject to 35 min preischemia, which included the required treatment as shown schematically in Figure 1. For IP-hearts, following a 20 min equilibration period hearts were subject to three cycles of 2 min global ischemia followed by 3 min reperfusion which we have shown to produce maximal protection\(^5\). When present, 50 µmol/L diazoxide or 200 nmol/L phorbol-12-myristate-13-acetate (TPA) were added 10 min before ischemia from 400 mmol/L and 100 mmol/L stock solutions in dimethyl sulfoxide respectively. Vehicle alone had no significant effect on the hemodynamic function or recovery of the heart from ischemia. Global normothermic ischemia (index ischemia) was induced for 30 min by halting perfusion and immersing the heart in perfusion buffer at 37°C. Normothermic perfusion was then restarted and continued for up to 60 min as required. Samples of effluent perfusate were collected for the spectrophotometric determination of lactate dehydrogenase (LDH) activity prior to ischemia and at 1, 5, 10, 15 and 30 min of reperfusion. At the required time during the perfusion protocols (Fig. 1), hearts were either rapidly homogenised for the preparation of mitochondria 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. In Supplementary Table 1 we present data on hemodynamic function and LDH release confirming that the protection of hearts by preconditioning protocols was similar to that observed previously\(^4\)\(^-\)\(^8\).

**Isolation and analysis of particulate and mitochondrial fractions**

All procedures were carried out at 0-4°C and are illustrated schematically in Supplementary Fig. 1s. For studies in which protein phosphorylation and protein kinase translocation were
to be studied, all buffers contained (mmol/L) sodium pyrophosphate 2, sodium fluoride 2 and β-glycerophosphate 2 plus phosphatase inhibitor cocktail 1 (Sigma, St. Louis, MO) and complete protease inhibitor cocktail (Roche Diagnostics, GmbH). Ventricles were rapidly cut away, weighed, and homogenized with a Polytron homogenizer at setting 3 for 5s in 5 ml of ice-cold sucrose buffer (mmol/L: sucrose 300, Tris-HCl 10, EGTA 2; pH 7.4). In some experiments a cytosolic fraction plus both a crude particulate fraction and a purer mitochondrial fraction were prepared as follows (protocol 1). The homogenate was rapidly diluted to 40 ml with isolation buffer containing 5mg/ml bovine serum albumin (BSA), centrifuged at 2000g for 90 s to remove cell debris and then centrifuged at 200,000g for 45 min to produce a crude total particulate fraction. A small sample of the supernatant (cytosol) and the pellet (crude particulate) were kept for analysis, whilst the remainder of the pellet was resuspended in 6 ml sucrose buffer containing 20% (v/v) Percoll and centrifuged at 12,000g for 10 min to yield a purified mitochondrial pellet. This was washed once in 6ml sucrose buffer followed by centrifugation at 12,000g. For a purer preparation of mitochondria, another procedure was used (protocol 2) that follows a normal mitochondrial preparation (known as crude mitochondria since there is contamination by plasma membranes) followed by Percoll gradient centrifugation to separate fractions containing pure plasma membranes and mitochondria. Following the initial homogenisation and 90 s centrifugation at 2000g the resulting supernatant was centrifuged at 10,000g for 5 min to yield a pellet containing crude mitochondria. This mitochondrial pellet was used without further purification for measurement of MPTP opening and protein carbonylation, whereas for the determination of protein kinases associated with the mitochondria, the pellet was resuspended in 6 ml isolation buffer containing 20% (v/v) Percoll and centrifuged at 14,000g for 10 min. This yielded a pellet containing purified mitochondria and a diffuse band at the top of the Percoll gradient containing plasma membranes that was removed for further analysis by SDS-PAGE (see
The mitochondrial pellet was washed once by resuspending in 6 ml isolation buffer before centrifugation at 12,000g for 5 min. For a more rapid isolation of membrane and particulate fractions, freeze-clamped, powdered tissue was extracted by a modified method of Hausenloy et al9,10. Briefly 50 mg powdered tissue per ml isolation buffer (containing protease and phosphatase inhibitors as above) were sonicated three times in 5 s bursts. Following centrifugation at 10,000 g for 10 s in a microcentrifuge to remove cell debris, the supernatant was centrifuged in the microcentrifuge at 10,000 g for 10 min to separate cytosol (supernatant) and particulate fractions (pellet). Cytosolic and particulate samples were dissolved in SDS-PAGE sample buffer, boiled at 100°C for 10 min and normalised to 1mg/ml using a Bicinchoninic acid-based (BCA) protein assay (Pierce).

**PKC translocation and protein phosphorylation determination**

Samples (10-25 µg protein) of the required sub-cellular fraction were separated by 10% SDS-PAGE (5% for determining acetyl-CoA carboxylase phosphorylation) using identical protein loading for each track. Gels were then subjected to western blotting with the required primary antibody and blots developed using anti-rabbit Ig horseradish peroxidase secondary antibody (anti-sheep Ig for total ACC protein), with ECL/ECL+ detection (Amersham Biosciences UK Limited). Appropriate protein loading and 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 was with AlphaEase v5.5 software. Each blot contained samples of all relevant fractions (usually cytosol, crude particulate and pure mitochondrial) from the control and preconditioned hearts (identical protein loading) to allow direct comparisons between groups using the same film exposure. For measurements of the phosphorylation state of Akt, GSK3β and AMPK, identical blots were performed in parallel and probed with primary antibody against the total kinase protein. Relative phosphorylation states were then
determined from the ratio of the phosphoprotein and total protein band intensities. PKC isoform translocation was determined from the ratio of the band intensity of the particulate sample to that of the cytosolic sample on the same blot. For the measurement of the relative mitochondrial and plasma membrane content of the different fractions parallel blots were performed on the same samples using antibodies against the adenine nucleotide translocase (ANT - a mitochondrial marker) and monocarboxylate transporter 1 (MCT1 - a specific plasma membrane marker).

Protein carbonylation assays

Protein carbonyls were analyzed according to Shacter\textsuperscript{12} as described previously\textsuperscript{5,8}. Briefly, an aliquot of the mitochondrial protein was derivatized with dinitrophenylhydrazine (DNPH - Chemicon International) under acid denaturing conditions. Proteins were separated by SDS-PAGE and subject to western blotting with anti-dinitrophenyl primary antibodies (Intergen Company, USA) at 1:1000 dilution. Each lane was loaded with an identical amount of protein (10 µg). In order to correct for non-specific binding of the antibodies, separate aliquots of the mitochondrial proteins that had been acid-denatured but not treated with DNPH were run in parallel. Blots were scanned and carbonylation determined as the sum of all band intensities for each track after subtraction of non-specific background signal).

Protein phosphorylation studies using 2D-gel electrophoresis

Samples for analysis by 2D-PAGE were precipitated using a 2D-clean-up kit (GE Healthcare) according to the manufacturer’s instructions and resuspended in buffer containing 7 mol/L urea, 2 mol/L thiourea, 40 g/L 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 60 mmol/L dithiothreitol, 5 g/L IPG Buffer pH3-11 NL (GE Healthcare) and 20 mg/L Bromophenol Blue. Samples (150 µg protein) were separated in the first dimension using pH 3-11 non-linear IPG strips (11 or 24 cm) on an Ettan IPGPhor and in the second
dimension using 12.5% acrylamide gels in an Ettan DALT Six Electrophoresis unit (both from GE Healthcare). Gels were stained for phosphoproteins using Pro-Q Diamond phosphoprotein stain (Invitrogen) and imaged using a Typhoon 9400 Variable Mode Imager (GE Healthcare). Gels were then stained for total protein using Sypro-Ruby protein gel stain (Invitrogen) and imaged again. The Pro-Q Diamond and Sypro Ruby images were overlaid using red and green channels in Adobe Photoshop (version 5.0). Where western blotting was also required, two gels were run in parallel to ensure accurate spot matching. For dephosphorylation of proteins, shrimp alkaline phosphatase (SAP from USB corporation) was employed.13 To 2 ml buffer (pH 7.9) containing (mmol/L) NaCl 10, Tris-HCl 5, MgCl₂ 1, dithiothreitol 0.1, Triton-X100 (1% w/v) and “complete” protease inhibitor cocktail (Roche) were added 0.8 mg mitochondrial protein and the mixed sample divided into 2 aliquots. To one aliquot (mock phosphatase treatment) was added 50 µl buffer containing 46 mmol/L each of sodium pyrophosphate, β-glycerophosphate and NaF. To the other aliquot were added 50 units of SAP. Both samples were incubated at 37°C for 30 min before addition of 250µl ice-cold trichloroacetic acid. The precipitated protein was then taken for proteomic analysis as above.

2-D fluorescence difference gel electrophoresis (DIGE)

Mitochondrial pellets were resuspended in 7 mol/L urea, 2 mol/L thiourea, 30 mmol/L Tris-HCl, and 4% (w/v) CHAPS, pH 8.5 at a concentration of 5-10 mg/ml and labeled for DIGE analysis using fluorescent cyanine dyes according to the manufacturer’s guidelines (GE-Healthcare). In brief, 50 µg of each sample was labeled with Cy3 or Cy5 N-hydroxysuccinamide (NHS) ester DIGE dyes freshly dissolved in anhydrous dimethylformamide. In each case, the labeling reaction was allowed to proceed on ice in the dark for 30 min. The reaction was terminated by the addition of 10 nmol lysine and subsequent incubation on ice in the dark for an additional 10 min. Cy3- and Cy5-labelled
samples were then combined, separated by 2D-PAGE as described above and scanned at two different wavelengths using a Typhoon 9400 variable mode imager to obtain images of the Cy3- and Cy5-labeled proteins.

**Measurement of MPTP opening in vitro**

The opening of the MPTP was determined at 25°C under de-energized conditions by following the decrease in light scattering (monitored as $A_{520}$) that accompanies mitochondrial swelling. Mitochondria were incubated for 2 min at 0.2 mg protein per ml in buffer (pH 7.2) containing (mmol/L): KSCN 150, Mops 20, Tris 10 and nitrilotriacetic acid 2, and supplemented with 2 µmol/L A23187, 0.5 µmol/L rotenone and 0.5 µmol/L antimycin A. Swelling was initiated by addition of 0.91 mmol/L CaCl$_2$ to give a buffered free $[Ca^{2+}]$ of 80 µmol/L$^{4,14}$.

**References**

5. Khaliulin I, Clarke SJ, Lin H, Parker J, Suleiman M-S, Halestrap AP. Temperature


**Supplementary Table 1** Effect of IP in the presence and absence of 10 µmol/L chelerythrine (CHE) or 10 µmol/L Compound C (CC) during 25 min index ischemia and after 60 min of reperfusion and on the extent of LDH release.

<table>
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<th>Parameters^a</th>
<th>Time to zero LVDP (min)</th>
<th>Time to start of contracture (min)</th>
<th>LVDP (mmHg)</th>
<th>HR (beat/min)</th>
<th>RPP (mmHg·beat/min)</th>
<th>LDH Release b (munits/min)</th>
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<tr>
<td>Pre-Ischemic</td>
<td>-</td>
<td>-</td>
<td>94.3 ± 3.2</td>
<td>301 ± 5.7</td>
<td>28299 ± 1025</td>
<td>57.3 ± 5.4</td>
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<tr>
<td>Values (n=40)</td>
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<tr>
<td>Control (n=8)</td>
<td>3.40 ± 0.14</td>
<td>13.08 ± 1.09</td>
<td>35.1 ± 6.7</td>
<td>100.8 ± 4.2</td>
<td>34.4 ± 5.6</td>
<td>56.4 ± 3.8, 47.5 ± 2.9, 35.9 ± 6.5</td>
</tr>
<tr>
<td>CC (n=6)</td>
<td>3.49 ± 0.11</td>
<td>12.43 ± 1.47</td>
<td>36.8 ± 9.6</td>
<td>96.3 ± 4.6</td>
<td>34.7 ± 7.5</td>
<td>41.5 ± 6.4, 33.7 ± 6.6, 31.3 ± 7.2</td>
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<td>CHE (n=6)</td>
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<td>11.75 ± 1.16</td>
<td>32.8 ± 3.9</td>
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<td>31.3 ± 4.0</td>
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<td>IP (n=8)</td>
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<td>62.5 ± 6.7</td>
<td>94.9 ± 4.9</td>
<td>58.5 ± 5.6 **</td>
<td>23.8 ± 2.2*, 29.6 ± 4.2, 27.5 ± 4.1</td>
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<td>IP + CC (n=6)</td>
<td>3.32 ± 0.16</td>
<td>7.47 ± 1.30 **</td>
<td>83.9 ± 14.2</td>
<td>88.9 ± 5.5</td>
<td>79.0 ± 13.9 *</td>
<td>24.4 ± 9.8*, 23.4 ± 7.0, 19.1 ± 5.1</td>
</tr>
<tr>
<td>IP + CHE (n=6)</td>
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<td>7.94 ± 1.03 **</td>
<td>66.0 ± 10.9</td>
<td>88.7 ± 3.5 *</td>
<td>57.6 ± 8.4 *</td>
<td>59.6 ± 9.1*, 55.8 ± 10.7*, 41.4 ± 6.7</td>
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^a All data are presented as means ± SEM. * P< 0.05, ** P< 0.01, *** P<0.001 vs. Control; # P<0.05, ## P<0.01, vs. corresponding group without CHE.

^b LDH release measured in perfusate collected for 5 min prior to ischemia and over the 3 times periods shown for reperfusion.
**Supplementary Table 2**  Characterization of proteins fractionated by 2D-PAGE and identified by MALDI-TOFTOF MS. The protein name and accession number represent the candidate proteins identified with the highest significant score from the MSDB database by the Mascot search engine. Protein score is defined as -10*Log(P), where P is the probability that the observed match is a random event and protein scores greater than 56 are significant (p<0.05). The theoretical isoelectric point (pI) and molecular weight (MW, Daltons) for each identified protein are shown. Where MSMS was performed, the precursor ion mass and resulting amino acid sequence are shown together with the corresponding ion score. Spots are identified by the Supplementary Figure number containing the relevant 2D gel and the area on the gel. In Fig. 7s several spots were picked within each area but in each case MS data are only presented for one spot since the others were identified as the same protein. Where data for the theoretic pI and MW are marked with an asterisk, values given correspond to the proteins with the mitochondrial targeting presequence removed.

<table>
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<tr>
<th>Spot No.</th>
<th>Protein Name</th>
<th>Accession number</th>
<th>Theoretical pl</th>
<th>Theoretical Mw</th>
<th>Protein Score</th>
<th>Sequence coverage</th>
<th>Number of matched peptides</th>
<th>Precursor ion mass</th>
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<td>NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial [precursor]</td>
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<td>Myosin light chain 1, slow-twitch muscle B/ventricular isoform</td>
<td>P16409</td>
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<td>22011.1</td>
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<td>P08733</td>
<td>4.86</td>
<td>18868.4</td>
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<td>EAFTIMDQNR</td>
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### Supplementary Table 2  continued - MS data for Fig 9s  Panel A

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<th>Protein Name</th>
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<th>Theoretical pI</th>
<th>Theoretical Mw</th>
<th>Protein Score</th>
<th>Sequence coverage</th>
<th>Number of matched peptides</th>
<th>Precursor ion mass</th>
<th>Peptide sequence</th>
<th>Ion score</th>
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<td>Pyruvate Dehydrogenase (lipoamide) (EC 1.2.4.1.) alpha Rattus norvegicus chain [precursor]</td>
<td>P26284</td>
<td>6.82*</td>
<td>40295.1*</td>
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<td>874.4781</td>
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<td>67</td>
<td>11.00%</td>
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<td><strong>Spot 3</strong></td>
<td>Ubiquinol-cytochrome-c reductase complex core protein I [precursor]</td>
<td>Q68FY0_RAT</td>
<td>5.22*</td>
<td>49380.6*</td>
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<td>49380.6*</td>
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<td>Voltage-dependent anion-selective channel protein isoform 1</td>
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<td>8.62</td>
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<td>43.50%</td>
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<td>1946.009</td>
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<td>SNFAVGYR VNNSSLIGVGYTQTL RPGVK</td>
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Legends to Supplementary Figures

**Figure 1s.** Protocols used for tissue fractionation (B). Details of the two procedures used for the preparation of mitochondria from perfused hearts and for cytosolic and particulate fractions from freeze clamped and powdered tissue are given in Supplementary Methods.

**Figure 2s** Ischemic preconditioning does not alter the sensitivity of MPTP opening to \([\text{Ca}^{2+}]\) in mitochondria isolated prior to ischemia. MPTP opening was measured under de-energised conditions using mitochondria isolated 5 min after the last ischemic phase of the IP protocol or from control hearts. The extent of MPTP opening following the addition of the free concentration of calcium shown was determined as the change in \(A_{520}\) as described under Supplementary Methods. Data are given as means ± S.E. of 6 separate mitochondrial preparations each assayed at both calcium concentrations.

**Figure 3s** The effects of preconditioning with diazoxide on the phosphorylation state of AMPK, Akt, GSK3β and ACC. Control hearts and those treated with 50 µM diazoxide for 10 min as shown in Fig. 1 were freeze-clamped and a cytosolic fraction produced according to the freeze-clamp protocol of Supplementary Fig. 1s. Proteins were separated by SDS-PAGE followed by western blotting with the appropriate antibody for the total (t) or phosphorylated (p) kinases indicated, or for acetyl-CoA carboxylase (ACC). Data are shown for two separate control and diazoxide hearts.

**Figure 4s** The effects of chelerythrine and compound C on the hemodynamic recovery and lactate dehydrogenase release of control and IP hearts subject to 30 min ischemia and 30 min reperfusion. Data are taken from Supplementary Table 1 where further details are given. The LDH release shown is the total release over the first 10 min of reperfusion.

**Figure 5s** Preconditioning does not change the phosphorylation state of mitochondrial proteins determined by 2D-gel electrophoresis. Mitochondria were isolated from control or IP hearts just prior to ischemia (Pre) or following 3 min reperfusion (Rep), separated by 2D gel electrophoresis...
and then stained with Pro-Q Diamond to preferentially stain phosphorylated proteins and then sypro-Ruby to stain all proteins. In panel A, data are the same as for Fig. 7 but the Pro-Q Diamond phosphoprotein stain (red) is overlaid on the Sypro-Ruby protein stain (green) to reveal those proteins preferentially stained with the Pro-Q Diamond and thus true phosphorylated proteins. The identity of spots within boxes 1 and 2 were established in the experiments reported in Fig. 9s and MS data are provided in Supplementary Table 2. In Panel B additional data from a separate experiment are shown that also contains data for mitochondria isolated at the end of ischemia.

**Figure 6s** *Preconditioning does change the phosphorylation state of some cytosolic proteins as determined by 2D gel electrophoresis.* Hearts were freeze-clamped before index ischemia either without preconditioning (Control), at the end of the last brief ischemic period (IP#) or after the subsequent 5 min perfusion that precedes index ischemia (IP). Cytosolic samples were prepared in the presence of protease and phosphatase inhibitors and proteins separated by 2D gel electrophoresis prior to staining with Pro-Q Diamond (phosphoprotein) and then Sypro-Ruby (total protein). The scanned images are overlaid with Pro-Q Diamond shown in red and sypro-Ruby in green. Areas showing difference in the Pro-Q Diamond staining are indicated.

**Figure 7s** *The use of 2-D fluorescence difference gel electrophoresis (DIGE) to compare proteins in mitochondria isolated from control and IP hearts.* Mitochondria were isolated from control and IP hearts before ischemia, at the end of 30 min ischemia or after 3 min reperfusion. Mitochondrial extracts were prepared and labelled with Cy3 (control) or Cy5 (IP) fluorescent cyanine dyes and then combined and separated by 2D gel electrophoresis using isoelectric focussing gels of 24 cm width as opposed to the 11 cm width employed in the phosphoproteome studies. Reciprocal labelling was also performed (as was the case for the data insets of heart set 2). Images of Cy3- and Cy5-labeled proteins were acquired at two separate wavelengths and overlaid so that spots which are more abundant in the control samples appear green, spots which are more abundant in the IP samples appear red and those which are unchanged between samples appear yellow. Boxes 1-3 show areas containing proteins exhibiting differences between control and IP mitochondria. However, these
Changes were not consistent between end ischemia and reperfusion, both of which showed inhibited IP opening, and were not reproducible between experiments as shown in the insets. Here data from two separate set of control and IP preischemic hearts are presented, with opposite fluorescent labelling. The identities of those proteins showing the largest changes in heart set 1 were determined by mass spectrometry as reported in Supplementary Table 2. In box 1 both red spots were found to be the Rieske iron sulphur protein of ubiquininol:cytochrome c reductase and in box 2 the two dominant green spots were found to be NADH:ubiquinine oxidoreductase subunit 10 (NDUFA-10) as were the yellow spots below. In box 3 the higher two redder spots were both myosin light polypeptide 3 (myosin light chain 1, slow-twitch muscle B/ventricular isoform) and the lower 2 spots both myosin regulatory light chain 2, ventricular/cardiac muscle isoform. Thus these spots in box 3 reveal minor variations in contamination of the mitochondrial fraction between different preparations.

**Figure 8s** Preconditioning does not change the phosphorylation state of mitochondrial proteins separated by 1D gel electrophoresis and revealed by Pro-Q Diamond staining or western blotting with phosphor-amino acid antibodies. The conditions were identical to those used for Fig. 3 of the main paper, but samples were separated by single dimensional SDS-PAGE and then either stained with Pro-Q Diamond (Panel A) or subject to western blotting with antibodies targeted to phosphoserine (Panel B), phosphothreonine (Panel C) or phosphotyrosine (Panel D). Note that the phosphoserine bands present at about 40 kDa and 30 kDa are likely to correspond to PDHE1α and VDAC1 + VDAC2 respectively as identified in Fig. 9s. As might be expected in Panels A and B, the intensity of the 40 kDa band decreased at the end of ischemia when dephosphorylation would be predicted as a result of the elevated [Ca²⁺] and low ATP levels that will activate PDHP phosphatase and inhibit PDH kinase.

**Figure 9s** Identification of phosphorylated subunits of PDHE1α, VDAC1 and VDAC2 In Panels A and B mitochondria isolated from control perfused hearts were either treated with shrimp alkaline
phosphatase as described under supplementary methods or subjected to a control incubation (Mock). Samples were separated on 2D gels with duplicate samples being run in parallel, one for staining with Pro-Q Diamond and then Sypro Ruby and the other used for Western blotting with PDHE1α (Panel A) and then VDAC (Panel B) antibodies. Only the relevant strip of the gel is shown since for the Western blots no other spots were visible. The spots shown with arrows show a decrease in intensity following phosphatase treatment in both the Pro-Q Diamond stained gel and the western blot. Where indicated, spots were picked and analysed by Mass Spectrometry and results are reported in Supplementary Table 2. In Panel A, spots 1 and 2 were identified as PDHE1α and spots 3 and 4 as ubiquinol-cytochrome-c reductase complex core protein I whose phosphorylation has been observed by others.15;16 In Panel B, spots 4 and 5 were identified as VDAC2 in non-phosphorylated and phosphorylated forms (theoretical pI 7.44 and 6.68 respectively). Spot 3 was identified as phosphorylated VDAC1 (pI 7.83) and spots 1 and 2 as non-phosphorylated VDAC1 (pI 8.62). In Panel C isolated heart mitochondria were incubated under energised conditions in the absence (Control) or presence (Calcium) of 1 µM [Ca^{2+}] to activate PDH phosphatase is situ before preparing samples for 2D gel separation and staining with Pro-Q Diamond. Note that the multiple spots corresponding to phosphorylated forms of PDH1Eα show a significant decrease relative to the adjacent two spots (ubiquinol-cytochrome-c reductase complex core protein I).
Perfused heart

Polytron homogenisation

Homogenate

10 min at 800g

Discard Pellet (nuclei + cell debris)

Clarified homogenate

5 min at 10,000g

Pellet = “crude” mitochondria

Supernatant discarded

Supernatant = cytosol

45 min at 200,000

Pellet = “pure” mitochondria

Percoll gradient centrifugation and wash of pellet

Pellet = crude particulate fraction

Mitochondrial protocol 2
Using conventional centrifuges

Freeze clamped powder

Sonication

Homogenate

10 s at 10,000g

Clarified homogenate

10 min at 10,000g

Supernatant = cytosol

Pellet = crude particulate fraction

Freeze-clamp protocol
Using microcentrifuge
Fig. 2S

Extent of swelling ($A_{520} \times 10^3$)

- 80 µM [Ca$^{2+}$]
- 150 µM [Ca$^{2+}$]

- Control
- IP
Fig. 3S
Fig. 4S

The graph shows the RPP (percent of preischemic value) and LDH release (units in 10 min) for different conditions: Control, IP, CHE, IP + CHE, CC, and IP + CC. The graph compares the preischemic and reperfused states for each condition.
Fig. 5S
Panel A

Control

Pre-ischemic

pH 3  pH 11

Reperfused 3 min

pH 3  pH 11

IP

250kDa  150kDa  100kDa  75kDa
50kDa  37kDa
25kDa  20kDa  15kDa

1  2

2  2

1  2

2  2

1  2
Fig. 5S Panel B

Control

IP

Pre ischemia  pH 3  pH 11

End ischemia  pH 3  pH 11

3 min Reperfusion  pH 3  pH 11

pH 3  pH 11  pH 3  pH 11  pH 3  pH 11

250kDa  150kDa  100kDa  75kDa  50kDa  37kDa  25kDa  20kDa  15kDa
Fig. 6S
Fig. 7S

Preischemic

End ischemic

3 min reperfusion

Heart set 1
Area 1
Area 2

Heart set 2
Area 1
Area 2

Area 1
Area 2
Area 3

Key:
- 250kDa
- 150kDa
- 100kDa
- 75kDa
- 50kDa
- 37kDa
- 25kDa
- 20kDa
- 15kDa
- 10kDa
Fig. 8S

A. ProQ Diamond stain

B. Anti-phospho-serine Ab

C. Anti-phospho-threonine Ab

D. Anti-phospho-tyrosine Ab
**Fig. 9S**

**A**

PDH α-subunit

- Western
- ProQ Diamond

- Mock
- Shrimp Pase

- 40 kDa
- 31 kDa

**B**

VDAC

- Western
- ProQ Diamond

- Mock
- Shrimp Pase

- pH 3
- pH 11

- 40 kDa
- 31 kDa

**C**

Control

- Calcium