Inhibition of mitochondrial permeability transition pore opening by ischemic preconditioning is probably mediated by reduction of oxidative stress rather than mitochondrial protein phosphorylation.

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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[1;2]. 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 PKCα, PKCδ and PKCε from Santa Cruz Biotechnology; against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Abcam; against total Akt, GSK3β and AMPK-α, and phosphorylated Akt (Ser473), GSK3α/β (Ser21/9) and AMPKα, (Thr172) from Cell Signaling Technology; against phosphorylated ACC (Ser79) from Upstate; against phosphothreonine and phosphoserine from Qiagen; against PDHE1α 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_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^{o}C (pH 7.4). Langendorff heart perfusions were performed as described previously[3;4] 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^{o}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^{o}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^{o}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

below). 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 al[9;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^{o}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[11]).

Protein carbonylation assays

Protein carbonyls were analyzed according to Shacter[12] as described previously[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, $MgCl_2$ 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 37ºC 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^{o}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].

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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.

Parameters [a]		Time to zero LVDP (min)	Time to start of contracture (min)		LVDP (mmHg)	HR (beat/min)	RPP (mmHg·beat/ min)	LDH Release [b] (munits/min)		
Pre-Ischemic Values (n=40)		-	-		94.3 ± 3.2	301 ± 5.7	28299 ± 1025	57.3 ± 5.4		
								Time of reperfusion		
					(% of initial values)			**5 min**	**10 min**	**15 min**
Control (n=8)	**Index ischemia**	3.40 ± 0.14	13.08 ± 1.09	**Post-ischemic**	35.1 ± 6.7	100.8 ± 4.2	34.4 ± 5.6	56.4 ± 3.8	47.5 ± 2.9	35.9 ± 6.5
CC (n=6)		3.49 ± 0.11	12.43 ± 1.47		36.8 ± 9.6	96.3 ± 4.6	34.7 ± 7.5	41.5 ± 6.4	33.7 ± 6.6	31.3 ± 7.2
CHE (n=6)		3.45 ± 0.02	11.75 ± 1.16		32.8 ± 3.9	94.8 ± 3.0	31.3 ± 4.0	38.4 ± 5.9	34.9 ± 7.1	28.6 ± 8.4
IP (n=8)		3.19 ± 0.06	5.81 ± 0.89 ***		62.5 ± 6.7 **	94.9 ± 4.9	58.5 ± 5.6 **	23.8 ± 2.2*	29.6 ± 4.2	27.5 ± 4.1
IP + CC (n=6)		3.32 ± 0.16	7.47 ± 1.30 **		83.9 ± 14.2 *	88.9 ± 5.5	79.0 ± 13.9 *	24.4 ± 9.8*	23.4 ± 7.0	19.1 ± 5.1
IP + CHE (n=6)		3.37 ± 0.12	7.94 ± 1.03 **		66.0 ± 10.9 *	88.7 ± 3.5 *	57.6 ± 8.4 *	59.6 ± 9.1##	55.8 ± 10.7#	41.4 ± 6.7

[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.

Spot No.	Protein Name	Accession number	Theoretical pI	Theoretical Mw	Protein Score	Sequence coverage	Number of matched peptides	Precursor ion mass	Peptide sequence	Ion score
Fig. 7s										
Area 1 spot A	Cytochrome bc1 complex subunit Rieske, mitochondrial precursor	AAH85339	9.04	29427.2	344	42.30%	10	816.509	GKPLFVR	43
								998.4578	VPDFSDYR	36
								1586.802	EIDQEAAVEVSQLR	84
Area 2 spot C	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial [precursor]	AAH93375	5.96*	36842.7	552	47.90%	11	1123.5895	LQSWLYASR	73
								2316.1006	SIYSDFVFLEAMYNQGFIR	43
								2441.3035	LLQYSDALEHLLSTGQGVVLER	102
								2657.3933	EVLNYTTVPVYLPEITIGAHQGSR	125
								2849.5447	LTLPEYLPPHAVIYIDVPVSEIQDR	58
Area 3 spot B	Myosin light chain 1, slow-twitch muscle B/ventricular isoform	P16409	5.03	22011.1	302	47.00%	9	1025.5051	EAFQLFDR	28
								1396.7543	ALGQNPTQAEVLR	61
								1501.6805	DTGTYEDFVEGLR	44
								1782.9497	AAPAPAAAPAAAPEPERPK	20
Area 3 spot D	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	P08733	4.86	18868.4	243	57.80%	9	1192.5957	DGFIDKNDLR	59
								1240.5626	EAFTIMDQNR	21

Cont/-

Supplementary Table 2 continued - MS data for Fig 9s Panel A

Spot No.	Protein Name	Accession number	Theoretical pI	Theoretical Mw	Protein Score	Sequence coverage	Number of matched peptides	Precursor ion mass	Peptide sequence	Ion score
Fig. 9s	**Panel A**									
Spot 1	Pyruvate Dehydrogenase (lipoamide) (EC 1.2.4.1.) alpha Rattus norvegicus chain [precursor]	P26284	6.82*	40295.1*	280	24.30%	12	874.4781	GDFIPGLR	8
								936.4686	AHGFTFTR	55
								1411.7792	LEEGPPVTTVLTR	83
Spot 2	Pyruvate Dehydrogenase (lipoamide) (EC 1.2.4.1.) alpha Rattus norvegicus chain 1 [precursor	P26284	6.82*	40295.1*	67	11.00%	6	936.4686	AHGFTFTR	10
Spot 3	Ubiquinol-cytochrome-c reductase complex core protein I [precursor]	Q68FY0_RAT	5.22*	49380.6*	171	20.63%	9	809.515	SLLTYGR	12
								1646.8132	EVESIGAHLNAYSTR	50
Spot 4	Ubiquinol-cytochrome-c reductase complex core protein I [precursor]	Q68FY0_RAT	5.22*	49380.6*	64	12.55%	5	809.5294	SLLTYGR	11

Cont/-

Supplementary Table 2 continued - MS data for Fig 9s Panel B

Spot No.	Protein Name	Accession number	Theoretical pI	Theoretical Mw	Protein Score	Sequence coverage	Number of matched peptides	Precursor ion mass	Peptide sequence	Ion score
Fig. 9s	**Panel B**									
1	Voltage-dependent anion-selective channel protein isoform 1	Q9Z2L0	8.62	30736.6	327	40.60%	8	1946.009	KLETAVNLAWTAGNSNTR	80
								2103.1809	VNNSSLIGLGYTQTLKPGIK	28
								2176.0518	WNTDNTLGTEITVEDQLAR	66
								2600.1899	TDEFQLHTNVNDGTEFGGSIYQK	16
2	Voltage-dependent anion-selective channel protein isoform 1	Q9Z2L0	8.62	30736.6	230	43.50%	8	1213.6221	VTQSNFAVGYK	32
								1400.6692	LTFDSSFSPNTGK	14
								2103.1809	VNNSSLIGLGYTQTLKPGIK	31
								2176.0518	WNTDNTLGTEITVEDQLAR	31
3	Voltage-dependent anion-selective channel protein isoform 1	Q9Z2L0	8.62	30736.6	237	43.50%	8	1946.009	KLETAVNLAWTAGNSNTR	50
								2103.1809	VNNSSLIGLGYTQTLKPGIK	34
								2176.0518	WNTDNTLGTEITVEDQLAR	35
4	Voltage-dependent anion-selective channel protein isoform 2	A38102	7.44	31699.6	156	24.70%	6	913.4526	SNFAVGYR	41
								2103.1555	VNNSSLIGVGYTQTLRPGVK	21
								2527.1848	TGDFQLHTNVNNGTEFGGSIYQK	14
5	Voltage-dependent anion-selective channel protein isoform 2	A38102	7.44	31699.6	101	9.50%	2	913.4526	SNFAVGYR	41
								2103.1555	VNNSSLIGVGYTQTLRPGVK	32

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 [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 [Ca^{2+}] and low ATP levels that will activate PDHP phosphatase and inhibit PDH kinase.

Figure 9s *Identification of phosphorylated subunits of PDHE1a , 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).

Fig. 1S

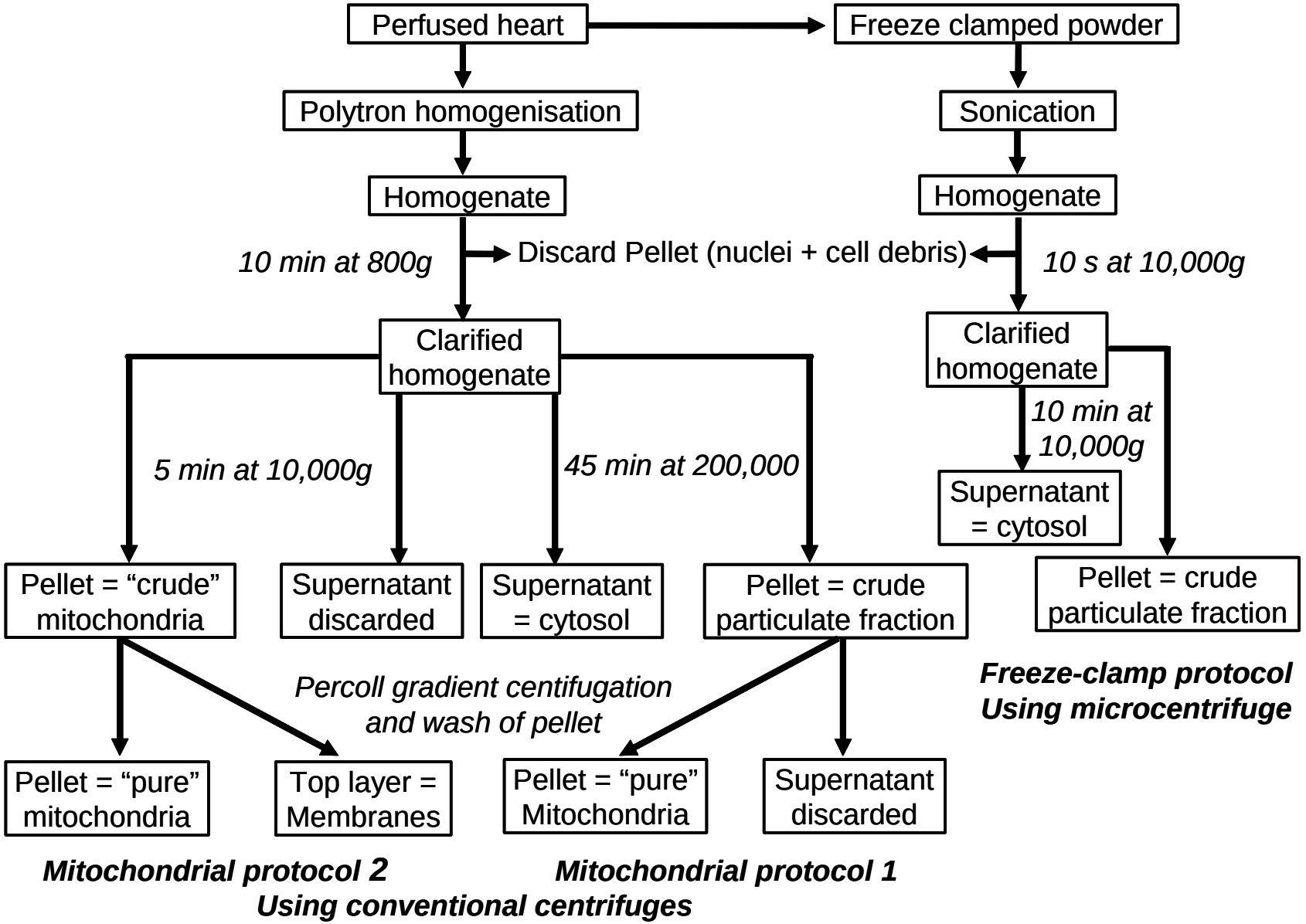

Perfused heart
Freeze clamped powder
Polytron homogenisation
Sonication
Homogenate
Homogenate
10 min at 800g
Discard Pellet (nuclei + cell debris)
10 s at 10,000g
Clarified homogenate
Clarified homogenate
10 min at 10,000g
5 min at 10,000g
45 min at 200,000
Supernatant = cytosol
Pellet = "crude" mitochondria
Supernatant discarded
Supernatant = cytosol
Pellet = crude particulate fraction
Pellet = crude particulate fraction
Freeze-clamp protocol
Using microcentrifuge
Percoll gradient centifugation and wash of pellet
Pellet = "pure" mitochondria
Top layer = Membranes
Pellet = "pure" Mitochondria
Supernatant discarded
Mitochondrial protocol 2
Mitochondrial protocol 1
Using conventional centrifuges

Fig. 2S

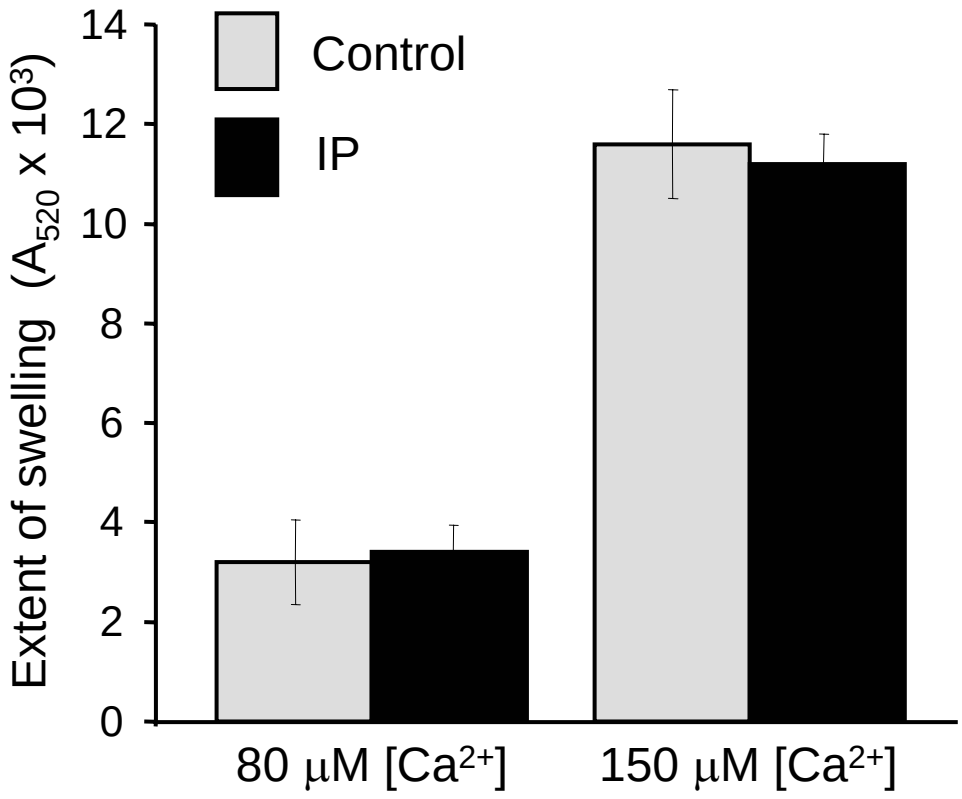

Control
IP
Extent of swelling (A520 x 10³)
14
12
10
8
6
4
2
0
80 μM [Ca2+]
150 μM [Ca2+]

Fig. 3S

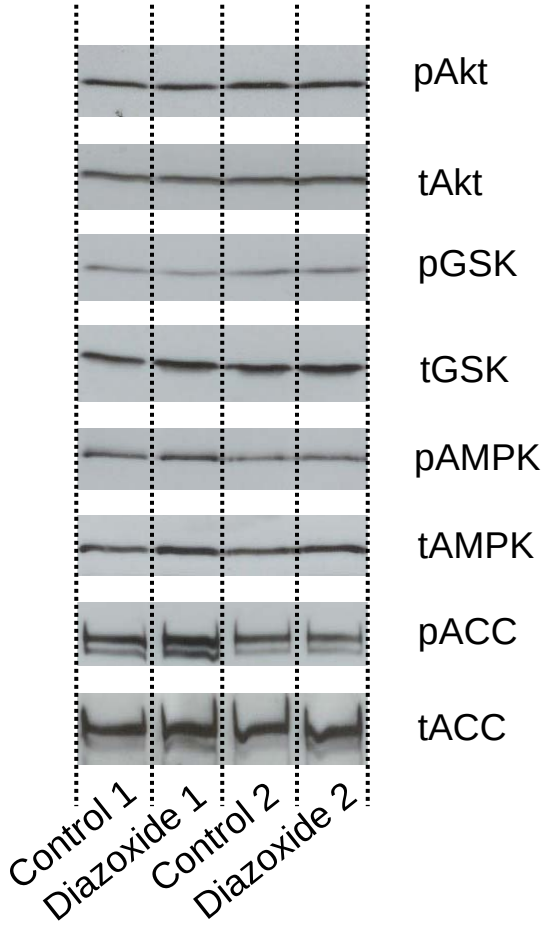

pAkt
tAkt
pGSK
tGSK
pAMPK
tAMPK
pACC
tACC
Control 1
Diazoxide 1
Control 2
Diazoxide 2

Fig. 4S

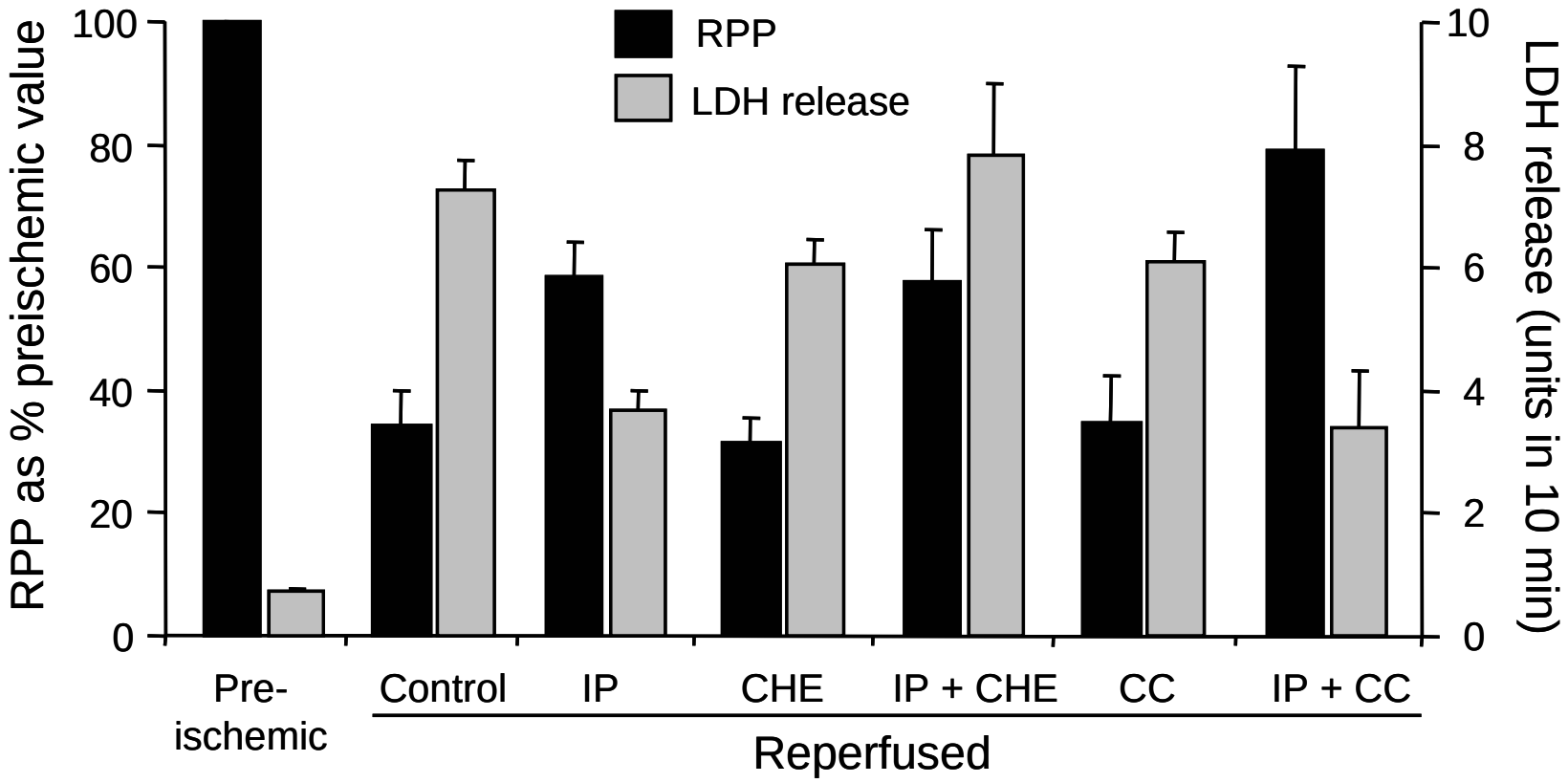

RPP
LDH release
RPP as % preischemic value
LDH release (units in 10 min)
100
80
60
40
20
0
10
8
6
4
2
0
Pre-
ischemic
Control
IP
CHE
IP + CHE
CC
IP + CC
Reperfused

Fig. 5S
Panel A

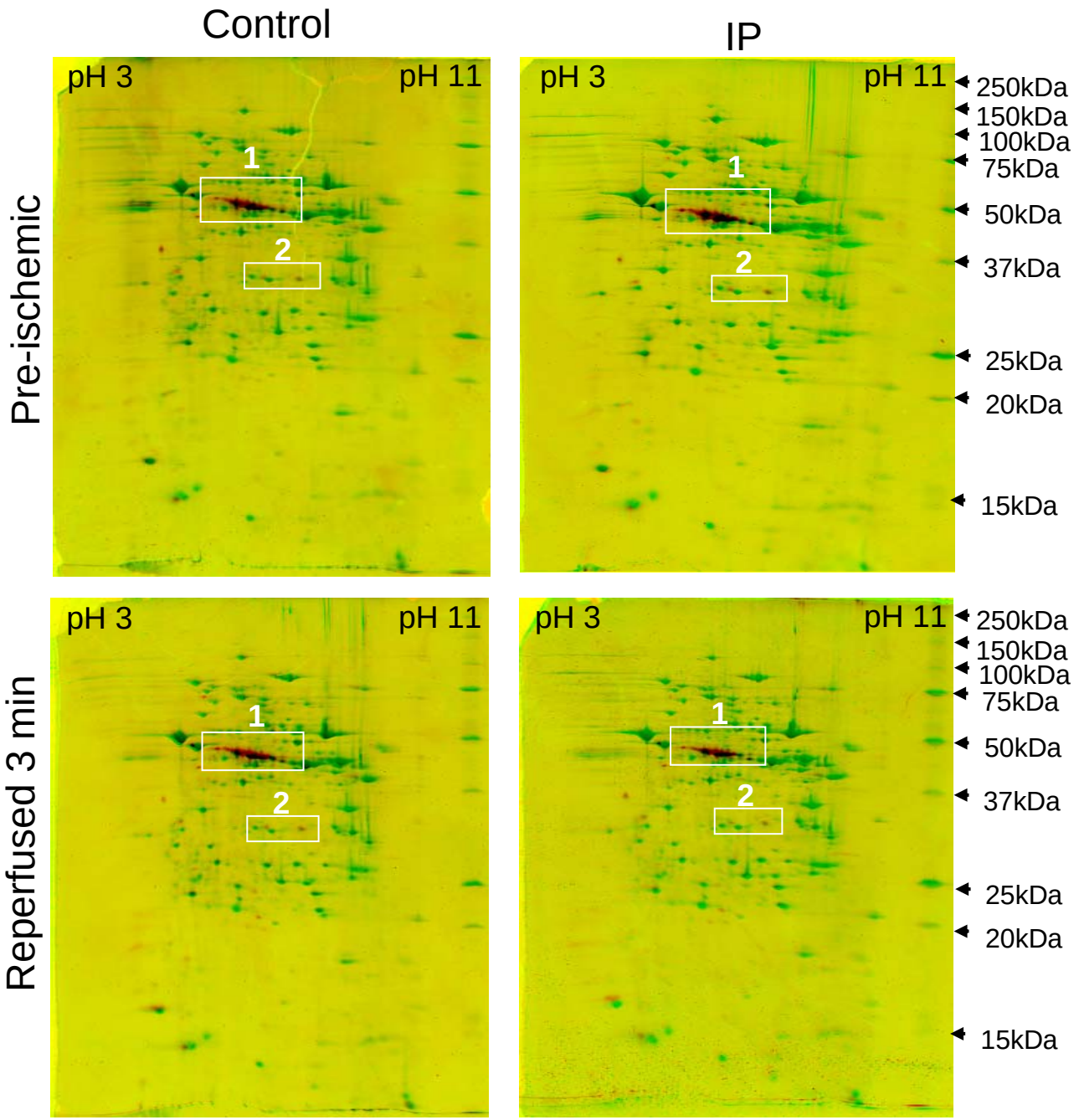


Fig. 5S
Panel B

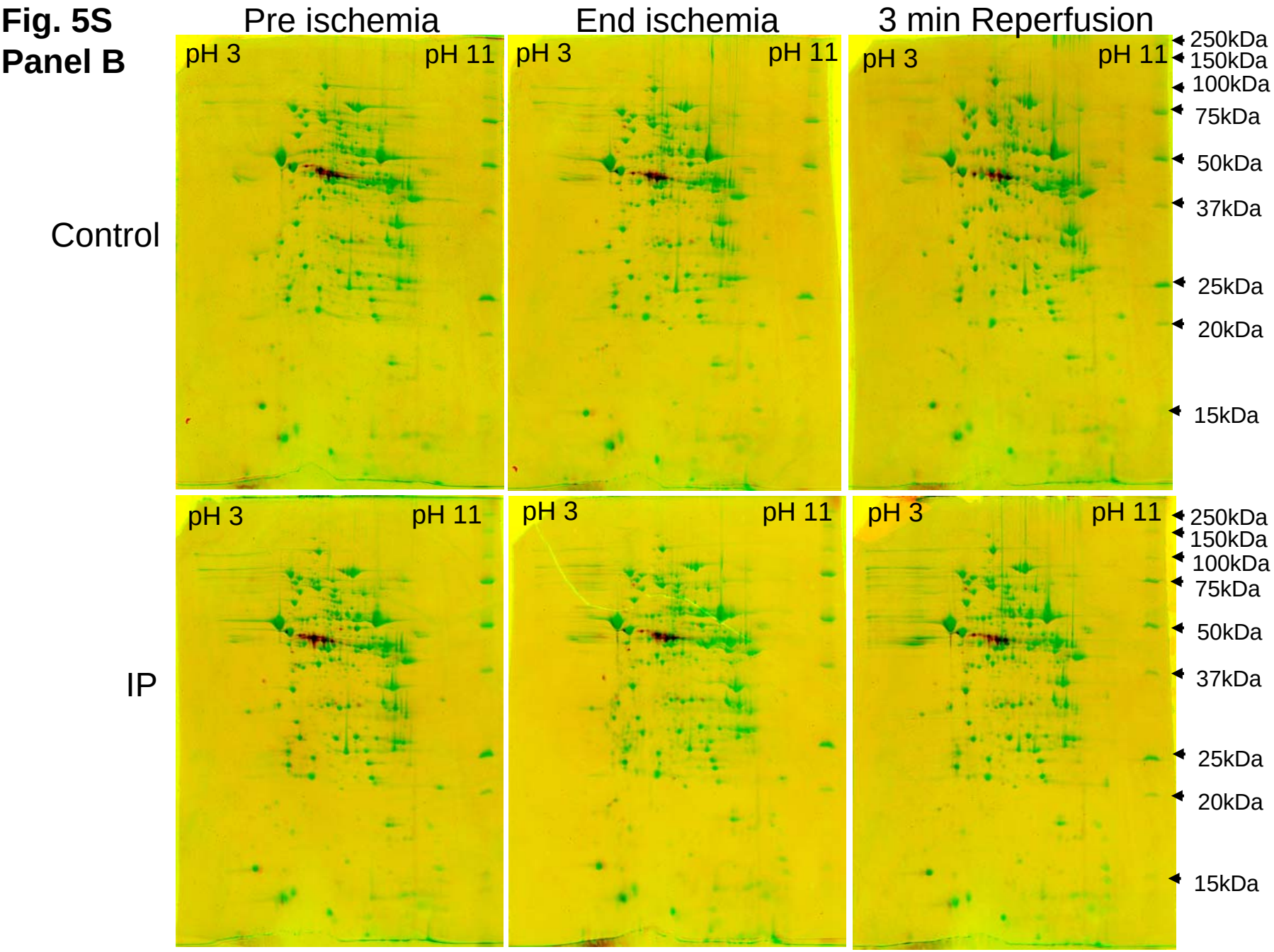


Fig. 6S

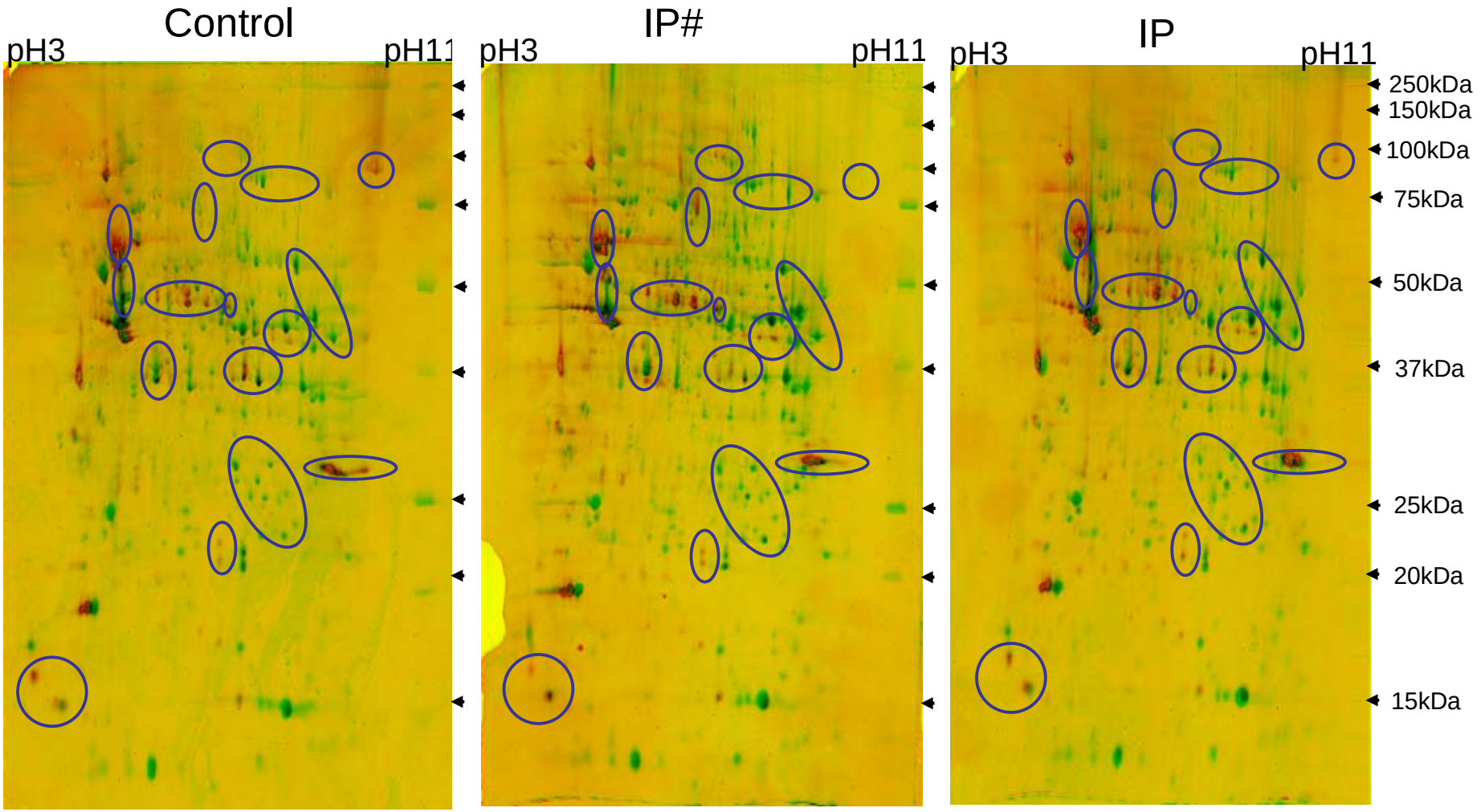

Control
pH3
pH11
IP#
pH3
pH11
IP
pH3
pH11
250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
25kDa
20kDa
15kDa

Fig. 7S

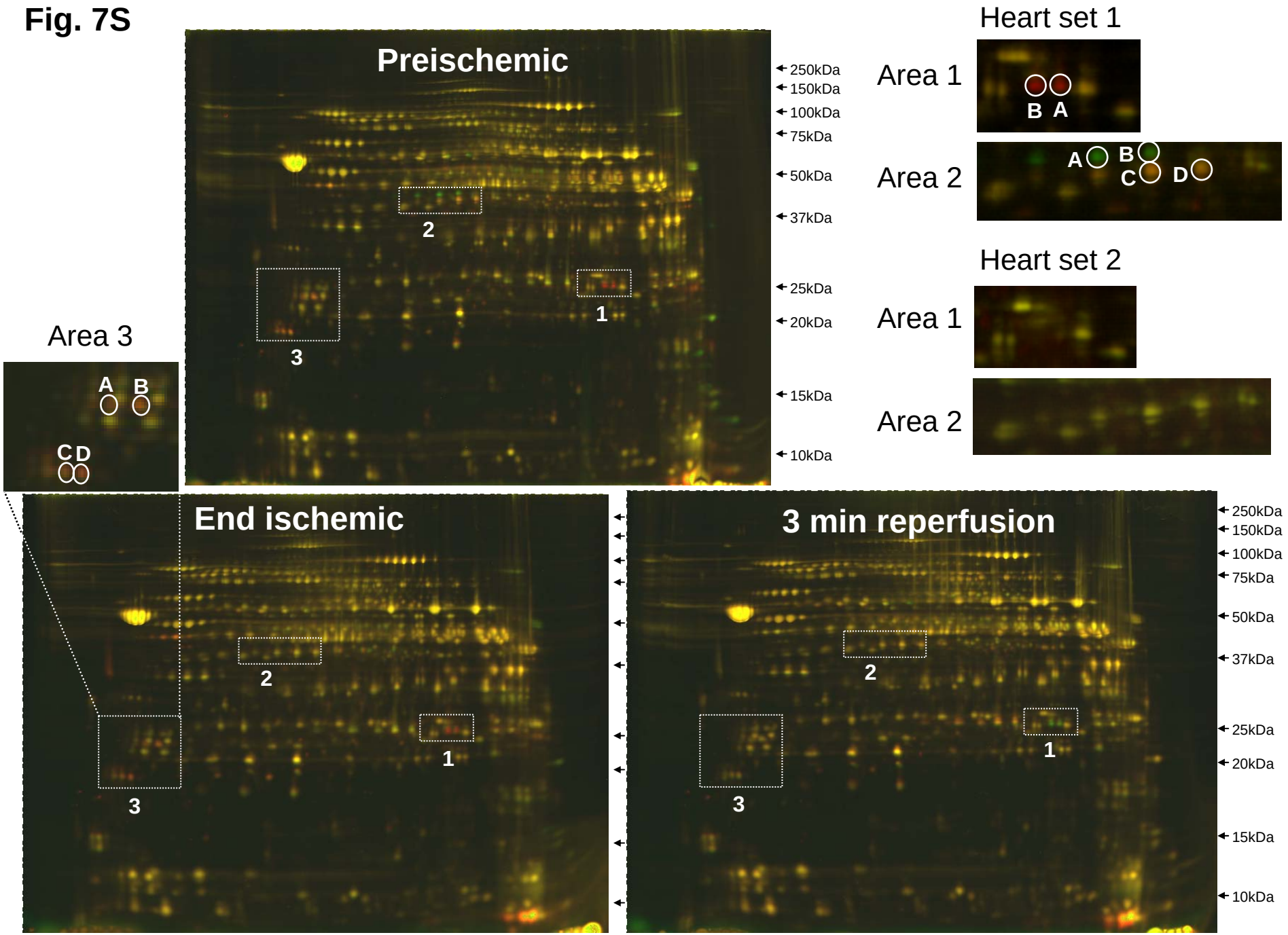

Preischemic
250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
25kDa
20kDa
15kDa
10kDa
1
2
3
Heart set 1
Area 1
B A
Area 2
A B C D
Heart set 2
Area 1
Area 2
Area 3
A B
C D
End ischemic
3 min reperfusion

Fig. 8S

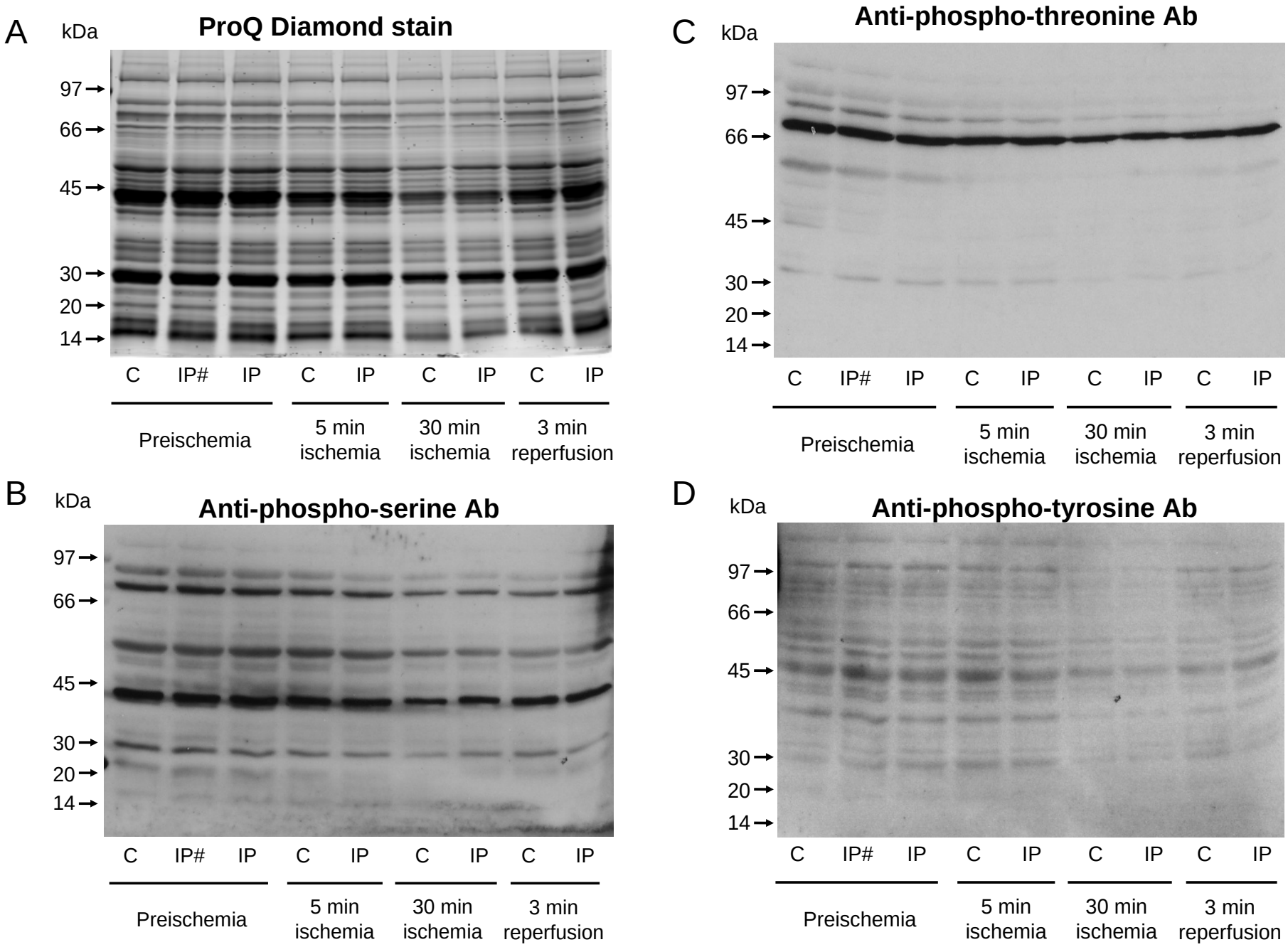
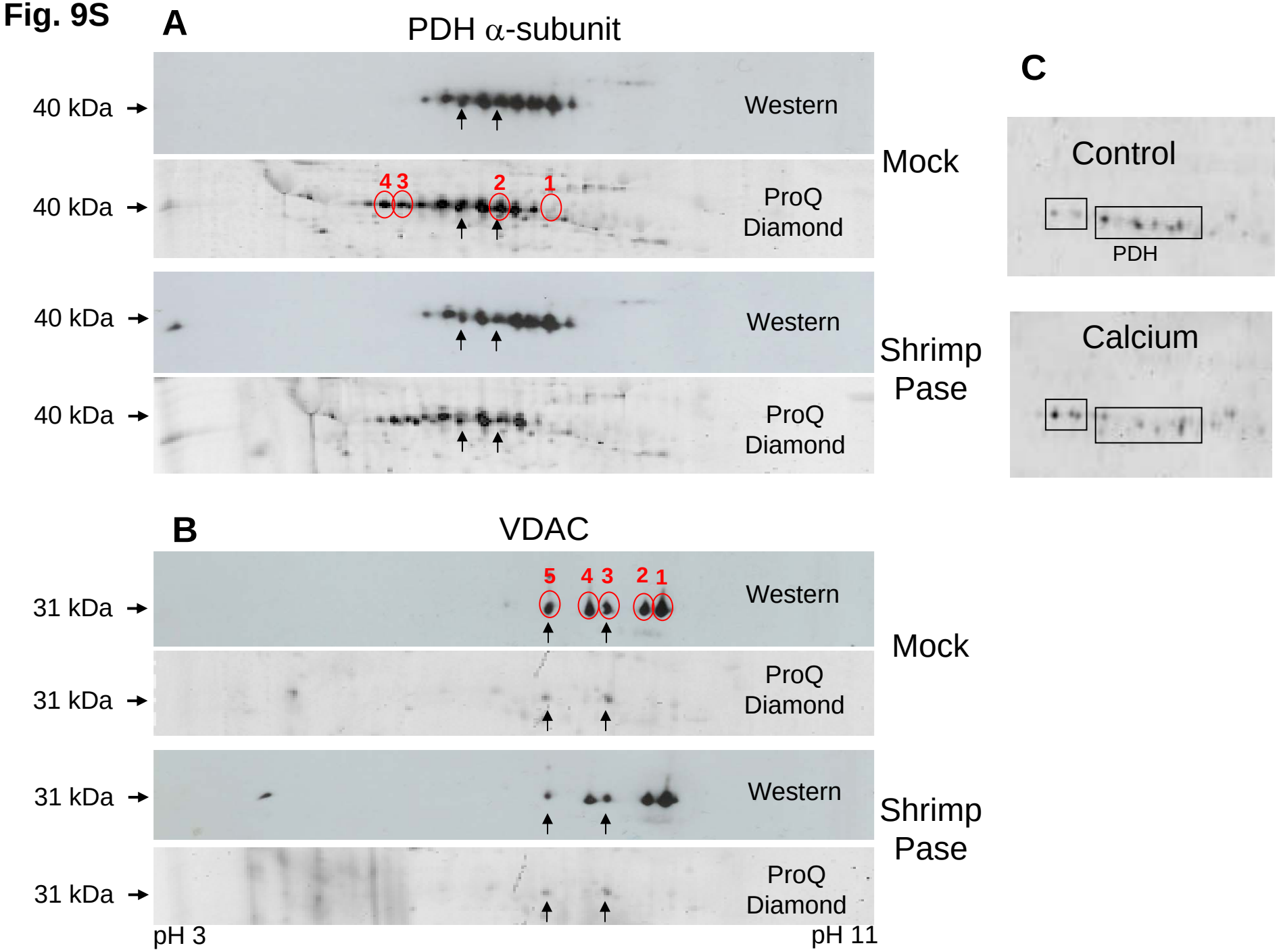

A
kDa
ProQ Diamond stain
97
66
45
30
20
14
C IP# IP C IP C IP C IP
Preischemia
5 min ischemia
30 min ischemia
3 min reperfusion
B
kDa
Anti-phospho-serine Ab
97
66
45
30
20
14
C IP# IP C IP C IP C IP
Preischemia
5 min ischemia
30 min ischemia
3 min reperfusion
C
kDa
Anti-phospho-threonine Ab
97
66
45
30
20
14
C IP# IP C IP C IP C IP
Preischemia
5 min ischemia
30 min ischemia
3 min reperfusion
D
kDa
Anti-phospho-tyrosine Ab
97
66
45
30
20
14
C IP# IP C IP C IP C IP
Preischemia
5 min ischemia
30 min ischemia
3 min reperfusion

Fig. 9S


A
PDH α-subunit
40 kDa
Western
4 3
2
1
ProQ Diamond
Mock
Shrimp Pase
B
VDAC
31 kDa
5 4 3 2 1
pH 3
pH 11
C
Control
PDH
Calcium