Cardioprotection by Ischemic Preconditioning Preserves Mitochondrial Function and Functional Coupling Between Adenine Nucleotide Translocase and Creatine Kinase

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[2]*Laboratory of Bioenergetics, Université Joseph Fourier B.P. 53X, 38041 Grenoble Cedex 9, France and* [3]*National Institute of Chemical and Biological Physics, Tallinn, Estonia, and* [4]*Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, Beaverton, OR 97006, USA*

M. N. Laclau, S. Boudina, J. B. Thambo, L. Tariosse, G. Gouverneur, S. Bonoron-Adèle, V. A. Saks, K. D. Garlid and P. Dos Santos. Cardioprotection by Ischemic Preconditioning Preserves Mitochondrial Function and Functional Coupling Between Adenine Nucleotide Translocase and Creatine Kinase. *Journal of Molecular and Cellular Cardiology* (2001) **33**, 947–956. This study investigates the effect of ischemic preconditioning on mitochondrial function, including functional coupling between the adenine nucleotide translocase and mitochondrial creatine kinase, which is among the first reactions to be altered in ischemia. Three groups of Langendorff-perfused rat hearts were studied: a control group, a group subjected to 30 min ischemia followed by 15 min reperfusion, and a group subjected to ischemic preconditioning prior to 30 min ischemia and 15 min reperfusion. Ischemic preconditioning significantly delayed the onset and amplitude of contracture during ischemia, decreased enzymatic release, and improved the recovery of heart contractile function after reperfusion. Mitochondrial function was assessed in permeabilized skinned fibers. The protective effect of preconditioning was associated with preservation of mitochondrial function, as evidenced by maintenance of the high $K_{1/2}$ for ADP in regulation of mitochondrial respiration and V_{max} of respiration, the near absence of respiratory stimulation by exogenous cytochrome c, and preservation of functional coupling between mitochondrial creatine kinase and adenine nucleotide translocase. These data suggest that ischemic preconditioning preserves the structure–function of the intermembrane space, perhaps by opening the mitochondrial ATP-sensitive K^+ channel. The consequence is preservation of energy transfer processes from mitochondria to ATP-utilizing sites in the cytosol. Both of these factors may contribute to cardioprotection and better functional recovery of preconditioned hearts. © 2001 Academic Press

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Introduction

The expression "ischemic preconditioning" (IPC) was introduced in 1986 by Murry *et al.*[1] to describe the apparently paradoxical finding that multiple brief episodes of ischemia and reperfusion prior to a sustained ischemic period reduced infarct size in dogs. IPC has been extensively investigated and verified in many

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animal species. The nature of the protection has also been extended to improved recovery of contractile function *in vivo* and *in vitro*,[2,3] decreased tissue damage and cell necrosis after reperfusion,[4] and delayed occurrence of ischemic contracture.[5–7]

Mitochondrial function is known to be altered by ischemia. Thus, the earliest detectable alterations during ischemia are a decrease in the functional coupling of mitochondrial creatine kinase (miCK) with the adenine nucleotide translocase (ANT) and an increase in the permeability of the mitochondrial outer membrane to ADP.[8–10] These alterations were observed after 15 min of ischemia at 37°C and persisted after reperfusion. In view of evidence for mitochondrial involvement, it is surprising that few studies[4,11] have examined whether IPC protects mitochondria function against ischemic damage.

This study employed isolated Langendorff-perfused rat hearts to examine the effects of IPC on cardiac function. Mitochondrial function was analyzed in permeabilized skinned fibers of the left ventricle prepared immediately at the end of the perfusion protocols. In agreement with previous results,[8–10] 30 min ischemia induced a decrease in the respiratory V_{max} and apparent $K_{1/2}$ of mitochondrial respiration for ADP [$K_{1/2}$ (ADP)] and a 66% decrease in functional coupling between ANT and miCK. The latter phenomenon reflects an alteration of energy transfer from the mitochondrial matrix to ATP-utilizing sites in the cytosol, and its reduction may contribute to the poor functional recovery of ischemic hearts. IPC prevented all of these changes in mitochondrial function.

The ischemia-induced alterations in mitochondrial function which were observed in these experiments are concentrated in the intermembrane space (IMS) and outer membrane of mitochondria. The beneficial effects of IPC are blocked by 5-hydroxydecanoate,[12,13] a specific inhibitor of the mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$),[14] indicating a role for mitoK$_{ATP}$ in cardioprotection.[15,16] We propose that IPC opens mitoK$_{ATP}$, leading to maintenance of IMS volume and preservation of the structure–function of this compartment. We conclude that IPC preserves mitochondrial function and energy transfer processes and thereby improves functional recovery after reperfusion.

Methods

Langendorff perfusions

Male Sprague–Dawley rats weighing 350–375 g (Elevage Janvier, Le Genest St Isle, France) were housed three per cage at constant temperature (22 ± 1°C) in environmental facilities with a 12 h light/dark cycle schedule and were given standard laboratory chow and tap water *ad libitum*. The rats were anesthetized with 40 mg sodium pentobarbital injected intraperitoneally. The thorax was opened, hearts were rapidly excised, immediately cooled in iced Krebs buffer and perfused by an aortic canula delivering warm (37°C), buffer at a constant pressure of 100 mmHg. Hearts were perfused with a modified phosphate-free Krebs–Henseleit solution containing 118 mM NaCl, 5.9 mM KCl, 1.75 mM CaCl$_2$, 1.2 mM MgSO$_4$, 0.5 mM EDTA, 25 mM NaHCO$_3$, 16.7 mM glucose. This high glucose concentration was employed to overcome possible limitations to glucose entry into the cardiomyocytes. The perfusate was gassed with 95%O$_2$–5%CO$_2$ which resulted in a pO$_2$ above 600 mmHg at the level of the aortic canula and a buffer pH of 7.4. The pulmonary artery was transected to facilitate coronary venous drainage, and a left ventricular polyethylene apical drain was inserted through a left atrial incision to allow Thebesien venous drainage. Left ventricular pressure was monitored from a water filled latex balloon placed through the left atrial appendage, and connected to a Statham P23Db pressure transducer. The volume of the balloon was adjusted to obtain a left ventricular diastolic pressure around 7 mmHg and kept constant throughout the entire experiment. Hearts were not paced and mechanical performance was evaluated as the product of heart rate and developed pressure (RPP).

Perfusion protocols

Three groups of hearts were studied ($n = 6$ in each group) in protocols described in Figure 1. The control group was perfused for 90 min under well-oxygenated conditions. The ischemia–reperfusion group (IR) was allowed to stabilize for 45 min under well-oxygenated conditions prior to 30 min of zero-flow ischemia followed by 15-min reperfusion. The preconditioned group (IPC) was allowed to stabilize for 15 min under well-oxygenated conditions before three cycles of 5-min zero-flow ischemia and 5-min reperfusion.[17] The IPC hearts were then submitted to 30 min of zero-flow ischemia followed by 15-min reperfusion. Left ventricular pressure and coronary flow were continually monitored. Coronary effluent was collected every min of the first 10 min of the reperfusion period to measure LDH and CPK release using the Olympus System Reagent OSR 6126[18] and 6179.[19]
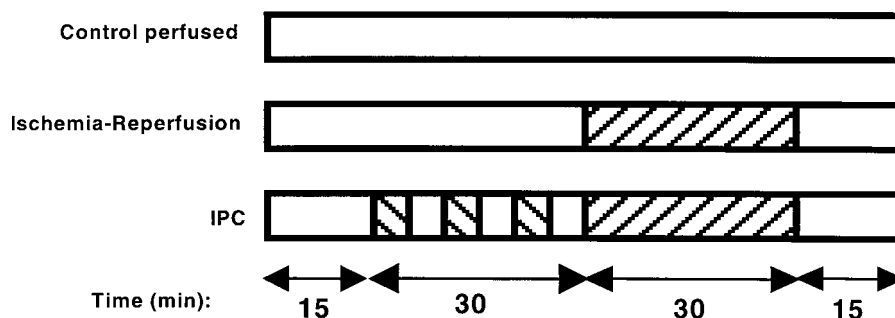
Figure 1 Schematic representation of the perfusion protocols. The numbers under the arrows represent the duration (in min) of each period. Perfused controls: 90 min of aerobic perfusion; Ischemia–reperfusion: 30 min of acute ischemia followed by 15 min reperfusion; IPC: ischemic preconditioning prior to 30 min of acute ischemia followed by 15 min reperfusion. □ Aerobic perfusion; ▨ preconditioning ischemia; ▨ ischemia.

Permeabilized cardiac fiber preparation

The permeabilized fiber technique, which has been extensively described,[9,20–22] was used to study mitochondrial function *in situ*. Scanning electron microscopy has shown that both subsarcolemmal and interfibrillar mitochondria are preserved.[23] The fact that this technique samples all of the mitochondria in the fiber is an important advantage, because it has been shown that subsarcolemmal mitochondria undergo a more rapid onset of ischemic damage.[24] Furthermore, the maximum respiration rate of the skinned fibers is equal to that expected on the basis of the mitochondrial content in heart tissue and respiration rates determined *in vitro* under the same experimental conditions.[20]

Small pieces of cardiac muscle were taken from left ventricle both at the end of the Langendorff-perfusion protocols and without prior perfusion (control *in situ* group) and put into cold (4°C) solution A. Solution A in mM: CaK_2EGTA 2.77; K_2EGTA 7.23 (pCa = 7); $MgCl_2$ 6.56; Dithiotheitol (DTT) 0.5; K-methanS 50; imidazole 20; taurine 20; Na_2ATP 5.3; and PCr 15. pH was adjusted to 7.1 at 25°C. These samples were rapidly dissected into bundles of fibers, which were incubated for 30 min with shaking in 1.8 ml solution A in the presence of saponin (50 μg/ml) in order to selectively destroy the sarcolemma. The bundles were subsequently put into solution B. Solution B in mM: CaK_2EGTA 2.77; K_2EGTA 7.23 (pCa = 7); $MgCl_2$ 1.38; DTT 0.5; K-methanS 50; imidazole 20; taurine 20; KH_2PO_4 3. pH was adjusted to 7.1 at 25°C twice for 10 min to wash out adenine nucleotides, phosphocreatine, and saponin. Oxygraphic measurements were performed in solution B supplemented with pyruvate 10 mM and malate 5 mM. Solutions A and B are based on the cytoplasmic composition of muscle cells. KCl solution was used

to test the intactness of the outer mitochondrial membrane. KCl solution in mM: KCl 125; HEPES 20; pyruvate 10; malate 5; Mg acetate 3; KH_2PO_4 5; EGTA 0.4; DTT 0.3. pH was adjusted to 7.1 at 25°C and 2 mg of bovine serum albumin per ml were added.

Respiration measurements

Respiratory rates of skinned fibers (0.5–0.75 mg DW) were determined using a Clark electrode in an oxygraphic cell containing 2 ml solution B supplemented with BSA or 2 ml KCl solution at 25°C with continuous stirring. The solubility of oxygen was estimated at 215 nmol O_2/ml.

Assessment of outer mitochondrial membrane integrity

Intactness of the outer membrane was determined in KCl medium, in which endogenous cytochrome c dissociates from the outer surface of the inner membrane but continues to support maximal respiration as long as the outer membrane remains intact.[25–27] We first measured the initial rate of respiration of skinned cardiac fibers in KCl solution containing substrates and no ADP (state 2). Then, respiration was stimulated by the addition of 1 mM ADP, which induced a maximum activation of respiration (state 3). Cytochrome c was added at a final concentration of 8 μM. If the outer membrane is intact, addition of exogenous cytochrome c has no effect on the respiratory rate. If the outer membrane is damaged, and if some cytochrome c was lost from mitochondria, the addition of cytochrome c increases the respiratory rate.[9]

Determination of ANT-miCK functional coupling

The respiratory rate of mitochondria in skinned cardiac fibers was measured in solution B. Increasing amounts of ADP ranging from 0.0125 to 1 mM were successively added. The stimulatory effect of ADP was calculated from the respiration rates measured in the presence of a given concentration of ADP minus the value in the absence of ADP (state 2). The half saturation constant for ADP [$K_{1/2}$ (ADP)] in the presence and absence of 20 mM creatine was calculated from double-reciprocal plots of the dependence of respiration on the concentration of ADP.

Functional coupling between ANT and miCK was expressed by the ratio:

$$(V_{max}/K_{1/2})_{Cr}/(V_{max}/K_{1/2})_{ADP}$$

Where $(V_{max}/K_{1/2})_{Cr}$ is the catalytic efficiency of stimulation of respiration by ADP in presence of 20 mM creatine and $(V_{max}/K_{1/2})_{ADP}$ is the catalytic efficiency of the stimulation of respiration by ADP in absence of creatine.

Statistical analysis of experimental data

All data are expressed as mean ± standard error of the mean. A two-way analysis of variance (ANOVA) for repeated measurements was performed to analyze hemodynamic parameters at different time points in the three different experimental conditions. Another two-way ANOVA was performed for comparison of LDH and CPK release during reperfusion. A single factor ANOVA was used to investigate respiration parameters. All ANOVA analyses led to highly significant differences. Mean values comparisons are performed by Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

Hemodynamic and enzyme release data

Our findings reproduce well-accepted data of cardioprotection by IPC, and will simply be summarized here. The control group showed a remarkable hemodynamic stability with less than 15% decrease in RPP over the 90-min perfusion period and a constant diastolic pressure.

The IR group exhibited cardiac arrest. Ischemic contracture was observed after 10 min of ischemia with a maximum of 36 ± 4 mmHg ($P < 0.01$ *v* control) reached at 25 min. Upon reperfusion, a discrete recovery of systolic function was observed with maximal RPP values at the end of the reperfusion period of 1400 ± 800 mmHg/min, corresponding to 4% of the RPP value at the beginning of the experiment. The poor early recovery of RPP is in agreement with the data of Yabe *et al.*[4] It was due primarily to incomplete recovery of developed pressure after 15 min reperfusion [10 ± 3 mmHg ($P < 0.01$ *v* control)] i.e. 9% of the starting developed pressure, but also to incomplete recovery of heart rate [135 ± 59 bpm ($P < 0.01$ *v* control)] i.e. 47% of the starting heart rate. These data show the early occurrence of contracture during ischemia and a poor early recovery of systolic function after reperfusion.

The IPC group exhibited a 30% decrease in RPP during the preconditioning period. Ischemic contracture was observed after 25 min of index ischemia, reaching a maximum value of 18 ± 4 mmHg at 30 min ($P < 0.01$ *v* IR). At reperfusion, heart function recovered with maximum RPP values of 11 400 ± 3600 mmHg/min ($P < 0.01$ *v* IR), corresponding to 33% of RPP recorded at the beginning of the protocol. This incomplete recovery of RPP after 15-min reperfusion was almost completely due to incomplete recovery of left ventricular developed pressure to 40 ± 11 mmHg, i.e. 35% of the starting developed pressure. It is to be noted that left ventricular diastolic pressure during reperfusion was significantly lower in IPC (46 ± 7 mmHg) compared to ischemic hearts (61 ± 3 mmHg) ($P < 0.01$). These data show that IPC significantly delayed the onset of ischemic contracture and decreased its amplitude. IPC also resulted in better functional recovery after reperfusion.

Thirty minute ischemia followed by reperfusion induced LDH and CPK release into the coronary effluent. This release was three-fold lower in the IPC group than in the ischemic group ($P < 0.01$). These data show more severe ischemia–reperfusion injury (necrosis) in the IR group than in the IPC group.

Effect of IPC on the integrity of the outer mitochondrial membrane

Figure 2 contains the results of the cytochrome c assay (See Methods) performed on control fibers and on fibers subjected to 30 min ischemia–reperfusion preceded or not by a preconditioning protocol (see Fig. 1).
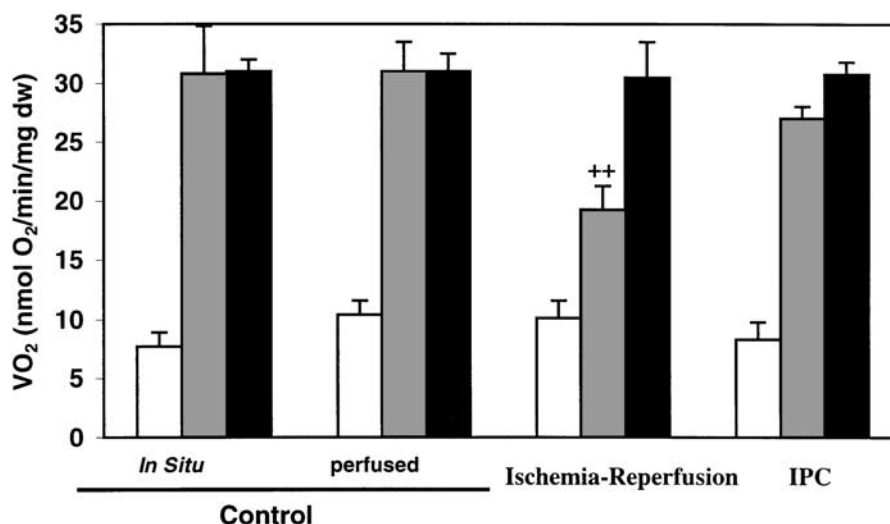
Maximum respiration rate was 31 ± 4 and

Figure 2 Effects of cytochrome c on mitochondrial respiration. The figure contains the results of oxygraphy measurements obtained in KCl solution, as described in (Methods). Oxygen consumption rates (nmol O_2 per minute per milligram dry weight) are shown for controls (white bars), in the presence of 1 mM ADP (gray bars), and in the presence of 1 mM ADP + 8 μM cytochrome c (black bars). Mitochondrial respiration was evaluated on permeabilized fibers obtained immediately after heart excision (*in situ* Control); from hearts subjected to 90 min normoxic Langendorff perfusion (Perfused Control); from hearts subjected to 30 min ischemia followed by 15 min reperfusion (ischemia–reperfusion); and from hearts subjected to the preconditioning protocol prior 30 min ischemia followed by 15 min reperfusion (IPC). + +$P < 0.01$ v control perfused.

31 ± 3 nmole O_2/min/mg dry weight (DW) in *in situ* and perfused control groups, respectively. This respiration was not stimulated by exogenous cytochrome c. There was an insignificant 25% increase in state 2 respiration in perfused *v in situ* controls hearts (10.4 and 7.7 nmole O_2/min/mg DW respectively; $P = 0.15$). However, respiratory rates recorded after inhibition of the ADP-stimulated respiration by oligomycin (1.5 μM) and atractyloside (50 μM) were the same in both control groups (data not shown). These data indicate that the perfusion protocol *per se* does not modify respiratory function or outer membrane integrity. The small increase in state 2 respiration in fibers obtained from perfused hearts may reflect increased proton leak in these mitochondria.

After 30-min ischemia followed by 15-min reperfusion, maximum respiration was significantly decreased by 38% compared to the control perfused group ($P < 0.01$). In these fibers, the addition of exogenous cytochrome c restored respiration to control values (Fig. 2). These data reflect alterations at the level of the outer mitochondrial membrane leading to a loss of endogenous cytochrome c. It is noteworthy that ischemia–reperfusion had no effect on state 2 respiration (Fig. 2).

In the IPC group, maximum respiration was significantly higher than in the ischemic group and decreased by only 13% compared to the control perfused group ($P < 0.01$). Addition of cytochrome

c in the KCl medium had only a minor effect on maximum respiration in the presence saturating concentrations of ADP, showing that the outer mitochondrial membrane was well preserved. These data indicate that IPC protects mitochondria against ischemia-induced outer membrane damage.

Regulation of respiration of permeabilized cardiac fibers by ADP and creatine

Figure 3 shows typical examples of double-reciprocal plots of the dependence of ADP-stimulated respiration rate on the concentration of ADP, in the presence or absence of creatine. Figure 4 contains a summary of results from the different experimental groups. Similar data from all experiments are statistically analysed in Figure 4.

In *in situ* and perfused control hearts, the $K_{1/2}$ (ADP) values for respiration (see Methods) were 395 ± 75 and 327 ± 45 μM, respectively (N.s.) [Fig. 4(A)]. These values declined in the presence of creatine to 65 ± 23 and 85 ± 24 μM in *in situ* and perfused controls, respectively (N.s.). As elaborated in the Discussion, the creatine-induced decrease in $K_{1/2}$ (ADP) reflects preservation of functional coupling in the intermembrane space between ANT and miCK.

In fibers from ischemic hearts, the $K_{1/2}$ (ADP) decreased dramatically to 92 ± 7 μM and decreased
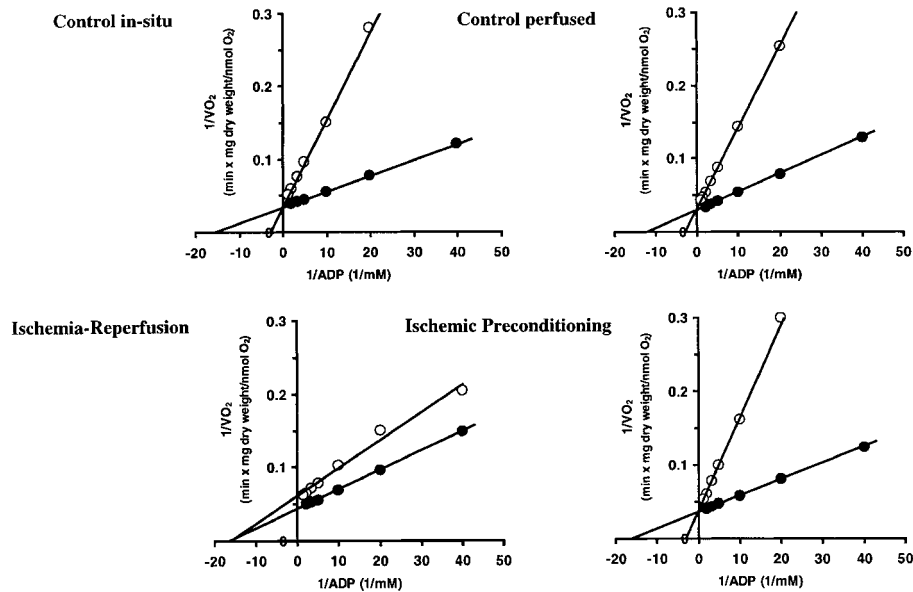
Figure 3 Dependence of respiration on ADP concentration. Shown are typical examples of double-reciprocal plots in the presence (black symbols) or absence (white symbols) of 20 mM creatine. Vo_2 is the ADP-stimulated respiration rate in nmol O_2 ($Vo_2 = Vo_{2\ ADP}-Vo_{2\ state\ 2}$). Experimental groups are defined as in Figure 2 (see also Fig. 1).

somewhat more in the presence of creatine to $64 \pm 7\ \mu M$, a value similar to that obtained in both control groups [Fig.4(A)]. This decline in $K_{1/2}$ (ADP) reflects a loss of adenine nucleotides compartmentation.

In sharp contrast with the data obtained from ischemic hearts, the $K_{1/2}$ (ADP) in fibers from preconditioned hearts remained high, with a value of $326 \pm 63\ \mu M$ [Fig. 4(A)]. This value is identical to the value of $327 \pm 45\ \mu M$ measured in fibers from control perfused hearts and reflects the low permeability of the outer mitochondrial membrane to ADP. Addition of creatine resulted in a decrease in $K_{1/2}$ (ADP) to $64 \pm 15\ \mu M$, a level identical to that observed in the control groups.

As presented in Figure 4(B), a quantitative estimation of this functional coupling can be obtained from the ratio of catalytic efficiency of the ADP stimulation of respiration in the presence and absence of creatine (see Methods). It can be seen that this parameter is significantly decreased by ischemia and well preserved when hearts are subjected to preconditioning prior to ischemia.

Discussion

This study demonstrates that IPC of Langendorff-perfused rat hearts improves recovery of cardiac function, decreases enzyme release after reperfusion, delays the onset of contracture, and decreases the amplitude of contracture. These

protective effects are in concordance with numerous reports in the literature.[1–7,17]

Our results also show, for the first time, that the protective action of IPC is associated with nearly complete preservation of mitochondrial function as it relates to energy transfer to the cytosol. We may identify four distinct effects of IPC that reflect protection of mitochondrial function: (1) reduced permeability to exogenous cytochrome c; (2) maintenance of high values of the $K_{1/2}$ (ADP) in regulation of respiration; (3) preservation of functional coupling between ANT and miCK; and (4) partial preservation of cytosolic ATP. The first three effects are documented in this paper, and the ATP-sparing effect of IPC was demonstrated by Murry *et al.*[28]

As shown in Figure 2, maximum respiration was significantly decreased after ischemia–reperfusion and was restored by exogenous cytochrome c. These data indicate that ischemia caused not only an increased outer membrane permeability to cytochrome c, but also a loss of cytochrome c from a fraction of mitochondria. This observation is in good agreement with data of Lesnefsky *et al.*[24] who showed a decrease in mitochondrial cytochrome c content during ischemia, and of Borutaite *et al.*,[26] who showed that the decrease in mitochondrial respiration after 30 min of ischemia is due to loss of cytochrome c. Most of this loss occurs after reperfusion,[29] but a small loss also occurs during ischemia.[24,30] It is not known whether the cytochrome c loss has a bearing on the mechanism of
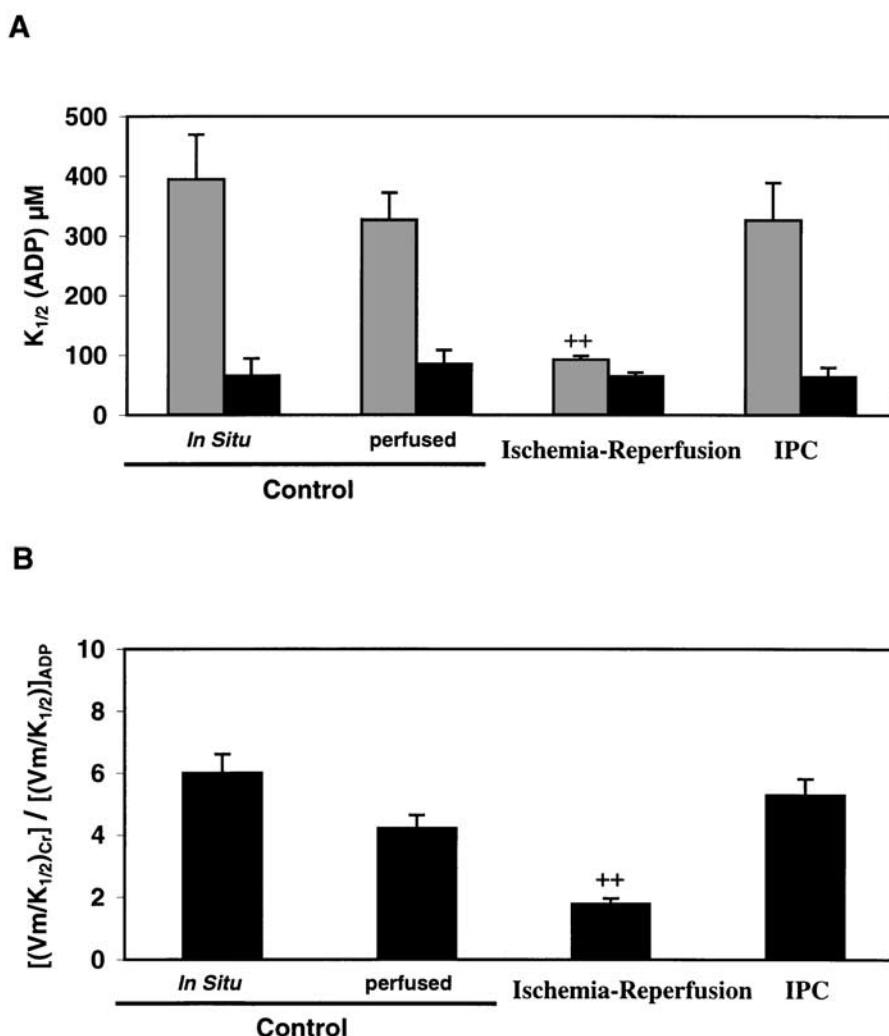
A

B

Figure 4 Control of oxidative phosphorylation in skinned fibers by ADP and creatine. (4A) $K_{1/2}$ (ADP) in the presence or absence of 20 mM creatine. The $K_{1/2}$ (ADP) was calculated from double-reciprocal plots of the dependence of ADP-stimulated respiration rate on the concentration of ADP, in the presence (black bars) or absence (gray bars) of 20 mM creatine. Experimental groups are defined as in Figure 2 (see also Fig. 1). (4B) Functional coupling between creatine kinase and oxidative phosphorylation. The ratio of the catalytic efficiency of the ADP-stimulated respiration in presence of creatine $(V_{max}/K_{1/2})_{Cr}$ v its catalytic efficiency in absence of creatine $(V_{max}/K_{1/2})_{ADP}$ shows the degree of stimulation of respiration by 20 mM creatine. $++P<0.01$ v control perfused.

apoptotic or necrotic cell death due to ischemia–reperfusion.[31,32] Respiratory stimulation by cytochrome c after ischemia-reperfusion is remarkable, because it occurs without any evidence of mitochondrial uncoupling. Thus, respiratory rates in both State 2 (no ADP) and State 3 (stimulated by ADP) are the same as in control. This means that the permeability of the outer membrane to cytochrome c is not associated with opening of the mitochondrial permeability transition[33] or other gross damage to mitochondria. In the IPC hearts, cytochrome c had little effect on maximum respiration, indicating that, whatever the mechanism

of the increased permeability to cytochrome c; it was prevented by IPC.

In the normal cardiomyocyte, efficient energy transfer between cytosol and mitochondria depends strongly on two organizational aspects of the mitochondrial isoenzyme of creatine kinase, which catalyzes the forward reaction:

$$Cr \ (creatine) + MgATP \rightarrow PCr \ (phosphocreatine) + MgADP.$$
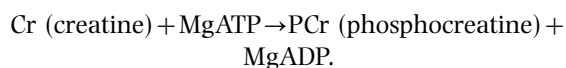
These two aspects are functional coupling and compartmentation, and both depend strongly on the

structure–function of the IMS. Functional coupling is the result of a close association between miCK and ANT, which is located in the inner mitochondrial membrane. Compartmentation refers to the normal low permeability of the outer membrane to ADP and ATP. Compartmentation may involve association of octameric miCK with outer membrane porin,[34] which causes porin to become poorly permeable to ADP and ATP (for review, see[22]).

In normal cardiac fibers, the low permeability of the outer membrane to ADP is reflected in the high $K_{1/2}$ for ADP-dependent respiration in control hearts, which is about $350\,\mu\text{M}$ [Fig. 4(A)]. Compartmentation is lost after ischemia, as reflected in the 3.5-fold drop in $K_{1/2}$ (ADP) and is preserved by IPC [Fig. 4(A)]. Functional coupling in normal cardiac fibers is reflected in the profound drop in the $K_{1/2}$ (ADP) in the presence of $20\,\text{mM}$ Cr [Fig. 4(A)], which occurs because creatine stimulates the production of ADP in the IMS, thereby reducing the influence of the outer membrane barrier to ADP [35]. This ratio decreased after ischemia–reperfusion; however, it must be noted that this decrease was strongly influenced by the loss of compartmentation and not solely by loss of functional coupling. When nucleotide compartmentation is lost after ischemia–reperfusion, respiration will be controlled by cytosolic ADP concentration, and restriction of ADP diffusion in the cytosol will likely result in a limitation of ATP production,[43] worsening ischemia-induced damage to the cardiomyocyte. An intact CK system, in which respiration is controlled primarily by ADP concentration in the intermembrane space, is necessary for the preservation of heart function and the achievement of the high work-state.[39–43] Thus, IPC protects against ischemia-induced changes in outer membrane permeability to ADP, and preserves the functional coupling between miCK and ANT [Fig. 4(A) and (B)].

One possible explanation for these beneficial effects of IPC is that IPC causes preservation of IMS volume and thereby preserves the structure–function of its components. Ischemia causes pathological contraction of the matrix and reciprocal swelling of the IMS, including the intercristal spaces.[36] At normal membrane potentials, mitochondria take up potassium by diffusion and expel potassium on the K^+/H^+ antiporter, thereby maintaining steady-state volume of matrix space and IMS. Ischemia causes a loss of membrane potential, due to anoxic blockade of electron transport. K^+ influx will all but cease, but K^+ efflux on the K^+/H^+ exchanger will continue until this process comes to a new steady state at a lower volume.[36,37] A

small contraction of the matrix will cause a much greater compensatory expansion of the IMS, because the surface area of the inner membrane is much greater than that of the outer membrane. Thus, the sequence of events during ischemia may occur in this way: Ischemia→depolarization→ matrix contraction due to net loss of K^+ and water →dissociation of miCK from outer and inner membranes→loss of compartmentation.

Opening the mitochondrial ATP-sensitive K^+ channel (mitoK$_{\text{ATP}}$) protects the heart against ischemia–reperfusion damage,[16] and mitoK$_{\text{ATP}}$ opening is thought to be involved in cardioprotection by IPC.[15,38] Moreover, it has been shown in heart mitochondria that the severe matrix contraction associated with inhibition of electron transport, as occurs during ischemia, can be prevented by the mitoK$_{\text{ATP}}$ opener, diazoxide.[36] Thus, opening mitoK$_{\text{ATP}}$ maintains the tight apposition of the inner and outer membranes of mitochondria. We propose that this effect preserves the structure–function of the IMS and prevents the changes caused by ischemia.

In conclusion, ischemic preconditioning preserves the integrity of the outer mitochondrial membrane and the structure–function of the mitochondrial intermembrane space, resulting in preservation of adenine nucleotide and cytochrome c compartmentation and functional coupling between ANT and miCK. It has been suggested that energy-sparing by IPC plays a major role in cardioprotection[11] and also that mitoK$_{\text{ATP}}$ is an end-effector of IPC.[15,38] Further studies are required to integrate these concepts and to test whether the beneficial effects of IPC arise from regulation of intermembrane space volume secondary to IPC-dependent opening of the mitochondrial ATP-sensitive potassium channel.

Acknowledgements

References

1. MURRY CE, JENNINGS RB, REIMER KA. Preconditioning with ischemia: a delay of lethal cell

injury in ischemic myocardium. *Circulation* 1986; **74**(5): 1124–1136.

2. ASIMAKIS GK, INNERS-McBRIDE K, MEDELLIN G, CONTI VR. Ischemic preconditioning attenuates acidosis and postischemic dysfunction in isolated rat heart. *Am J Physiol* 1992; **263**(3 Pt 2): H887–H894.

3. ASIMAKIS GK, ZWISCHENBERGER JB, INNERS-McBRIDE K, SORDAHL LA, CONTI VR. Postischemic recovery of mitochondrial adenine nucleotides in the heart. *Circulation* 1992; **85**(6): 2212–2220.

4. YABE K, NASA Y, SATO M, IIJIMA R, TAKEO S. Preconditioning preserves mitochondrial function and glycolytic flux during an early period of reperfusion in perfused rat hearts. *Cardiovasc Res* 1997; **33**(3): 677–685.

5. JANIER MF, VANOVERSCHELDE JL, BERGMANN SR. Ischemic preconditioning stimulates anaerobic glycolysis in the isolated rabbit heart. *Am J Physiol* 1994; **267**(4 Pt 2): H1353–H1360.

6. ARAD M, DE JONG JW, DE JONGE R, HUIZER T, RABINOWITZ B. Preconditioning in globally ischemic isolated rat hearts: effect on function and metabolic indices of myocardial damage. *J Mol Cell Cardiol* 1996; **28**(12): 2479–2490.

7. DEKKER LR, FIOLET JW, VANBAVEL E, *et al.* Intracellular Ca2+, intercellular electrical coupling, and mechanical activity in ischemic rabbit papillary muscle. Effects of preconditioning and metabolic blockade. *Circ Res* 1996; **79**(2): 237–246.

8. KAY L, SAKS VA, ROSSI A. Early alteration of the control of mitochondrial function in myocardial ischemia. *J Mol Cell Cardiol* 1997; **29**(12): 3399–3411.

9. KAY L, ROSSI A, SAKS V. Detection of early ischemic damage by analysis of mitochondrial function in skinned fibers. *Mol Cell Biochem* 1997; **174**(1–2): 79–85.

10. KAY L, DANESHRAD Z, SAKS VA, ROSSI A. Alteration of mitochondrial respiration by outer mitochondrial membrane and creatine during heart preservation. *Cardiovasc Res* 1997; **34**: 547–556.

11. REIMER K. The slowing of ischemic energy demand in preconditioned myocardium. *Ann NY Acad Sci* 1996; **793**: 13–26.

12. AUCHAMPACH JA, GROVER GJ, GROSS GJ. Blockade of ischaemic preconditioning in dogs by the novel ATP dependent potassium channel antagonist sodium 5-hydroxydecanoate. *Cardiovasc Res* 1992; **26**: 1054–1062.

13. HIDE EJ, THIEMERMANN C. Limitation of myocardial infarct size in the rabbit by ischaemic preconditioning is abolished by sodium 5-hydroxydecanoate. *Cardiovasc Res* 1996; **31**: 941–946.

14. JABUREK M, YAROV-YAROVOY V, PAUCEK P, GARLID KD. State-dependent inhibition of the mitochondrial K_{ATP} channel by glyburide and 5-hydroxydecanoate. *J Biol Chem* 1998; **273**: 13578–13582.

15. GROVER GJ, GARLID KD. ATP-sensitive potassium channels: a review of their cardioprotective pharmacology. *J Mol Cell Cardiol* 2000; **32**: 677–695.

16. GARLID KD, PAUCEK P, YAROV-YAROVOY V, *et al.* Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K+ channels: possible mechanism of cardioprotection. *Circ Res* 1997; **81**: 1072–1082.

17. SCHULTZ JE, YAO Z, CAVERO I, GROSS GJ. Glibenclamide-induced blockade of ischemic preconditioning is time dependent in intact rat heart. *Am J Physiol* 1997; **272**: H2607–H2615.

18. COMMITTEE oE: Recomended methods for the determination of four enzymes in blood. *Scand J Lab Invest* 1974; 33: 291–306.

19. HORDER M, JORGENSEN PJ, HAFKENSCHEID JC. Creatine kinase determination: a european evaluation of the creatine kinase determination in serum, plasma and whole blood with the Reflotron system. *Eur J Clin Biochem* 1991; **29**: 691–696.

20. VEKSLER VI, KUZNETSOV AV, SHAROV VG, KAPELKO VI, SAKS VA. Mitochondrial respiratory parameters in cardiac tissue: a novel method for assessment by using saponin-skinned fibers. *Biochim Biophys Acta* 1987; **892**: 191–196.

21. SAKS VA, VASILYEVA EV, BELIKOVA YO, *et al.* Retarded diffusion of ADP in cardiomyocytes: possible role of mitochondrial outer membrane and creatine kinase in cellular regulation of oxydative phosphorylation. *Biochim Biophys Acta* 1993; **1144**: 134–148.

22. SAKS VA, VEKSLER VI, KUZNETSOV AV, *et al.* Permeabilized cell and skinned fiber techniques in studies of mitochondrial function *in vivo*. *Mol Cell Biochem* 1998; **184**: 81–100.

23. SAKS VA, BELIKOVA YO, KUZNETSOV AV. *In vivo* regulation of mitochondrial respiration in cardiomyocytes: specific restrictions for intracellular diffusion of ADP. *Biochim Biophys Acta* 1991; **1074**: 302–311.

24. LESNEFSKY EJ, TANDLER B, YE J, SLABE TJ, TURKALY JS, HOPPEL CL. Myocardial ischemia decreases oxidative phosphorylation through cytochrome oxidase in subsarcolemmal mitochondria. *Am J Physiol* 1997; **273**: H1544–H554.

25. KLINGENBERG M, PFAFF E. Structural and functional compartmentation in mitochondria. In: Proceedings of the Symposium on Regulation of Metabolic Processes in Mitochondria. Tager JM, Papa S, Quagliariello E, Slater EC, eds. Amsterdam: Elsevier publishing company, 1965: 180–201.

26. BORUTAITE V, MORKUNIENE R, BUDRIUNAITE A, *et al.* Kinetic analysis of changes in activity of heart mitochondrial oxidative phosphorylation system induced by ischemia. *J Mol Cell Cardiol* 1996; **28**(10): 2195–2201.

27. HACKENBROCK CR, CHAZOTTE B, GUPTE SS. The random collision model and a critical assessment of diffusion and collision in mitochondrial electron transport. *J Bioenerg Biomembr* 1986; **18**: 331–363.

28. MURRY CE, RICHARD VJ, REIMER KA, JENNINGS RB. Ischemic preconditioning slows inergy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res* 1990; 66: 913–931.

29. DI LISA F, MENABO R, CANTON M, PETRONILLI V. The role of mitochondria in the salvage and the injury of the ischemic myocardium. *Biochim Biophys Acta* 1998; **1366**(1–2): 69–78.

30. SAKS VA, KAY L, ROSSI A. Novel method for evaluation of efficiency of heart preservation and intraoperational protection by analysis of the respiration of skinned fibers. 6th international symposium on cardiac surgery, Rome, 1995.

31. Gottlieb RA, Engler RL. Apoptosis in myocardial ischemia-reperfusion. *Ann N Y Acad Sci* 1999; **874**: 412–426.

32. Elsasser A, Suzuki K, Schaper J. Unresolved issues regarding the role of apoptosis in the pathogenesis of ischemic injury and heart failure. *J Mol Cell Cardiol* 2000; **32**(5): 711–724.

33. Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 1999; **341**(Pt 2): 233–249.

34. Schlattner U, Wallimann T. Octamers of mitochondrial creatine kinase isoenzymes differ in stability and membrane binding. *J Biol Chem* 2000; **275**: 17314–17320.

35. Saks VA, Kapelko VI, Kupriyanov VV, *et al.* Quantitative evaluation of relationship between cardiac energy metabolism and postischemic recovery of contractile function. *J Mol Cell Cardiol* 1989; **21**: 67–78.

36. Kowaltowski AJ, Seetharaman S, Paucek P, Garlid KD. Bioenergetic consequences of opening the mitochondrial ATP-sensitive K$^+$ channel of heart mitochondria. *Am J Physiol Heart Circ Physiol* 2001; **280**(2): H649–H657.

37. Garlid KD. Cation transport in mitochondria—the potassium cycle. *Biochim Biophys Acta* 1996; **1275**(1–2): 123–126.

38. Nakano A, Cohen MV, Downey JM. Ischemic preconditioning. From basic mechanisms to clinical applications. *Pharmacol Ther* 2000; **86**: 263–275.

39. Ingwall JS. Is cardiac failure a consequence of decreased energy reserve? *Circulation* 1993; **87** (Suppl. VII): VII-58–VII-62.

40. Saupe KW, Spindler M, Tian R, Ingwall JS. Impaired cardiac energetics in mice lacking muscle-specific isoenzymes of creatine kinase. *Circ Res* 1998; **82**(8): 898–907.

41. Tian R, Ingwall JS. Energetic basis for reduced contractile reserve in isolated rat hearts. *Am J Physiol* 1996; **270**: H1207–H1216.

42. Van Dorsten FA, Nederhoff MGJ, Nicolay K, Van Echteld CJA. P-31 NMR studies of creatine kinase flux in M-creatine kinase-deficient mouse heart. *Amer J Physiol Heart Circ Phy* 1998; **44**(4): H1191–H1199.

43. Dos Santos P, Aliev MK, Diolez P, Duclos F, Besse P, Bonoron-Adèle B, Sikk P, Canioni P, Saks VA. Bioenergetic control of contractile performance in isolated rat heart. Analysis of experimental data by reaction:diffusion mathematical model. *J Mol Cell Cardiol* 2000; **32**: 1703–1734.