Preconditioning protects by inhibiting the mitochondrial permeability transition

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Preconditioning protects by inhibiting the mitochondrial permeability transition. Am J Physiol Heart Circ Physiol 287: H841–H849, 2004. First published April 8, 2004; 10.1152/ajpheart.00678.2003.—Mitochondrial permeability transition (mPT) is a crucial event in the progression to cell death in the setting of ischemia-reperfusion. We have used a model system in which mPT can be reliably and reproducibly induced to test the hypothesis that the profound protection associated with the phenomenon of myocardial preconditioning is mediated by suppression of the mPT. Adult rat myocytes were loaded with the fluorescent probe tetramethylrhodamine methyl ester, which generates oxidative stress on laser illumination, thus inducing the mPT (indicated by collapse of the mitochondrial membrane potential) and ATP depletion, seen as rigor contracture. The known inhibitors of the mPT, cyclosporin A (0.2 μM) and N-methyl-4-valine-cyclosporin A (0.4 μM), increased the time taken to induce the mPT by 1.8- and 2.9-fold, respectively, compared with control (P < 0.001) and rigor contracture by 1.5-fold compared with control (P < 0.001). Hypoxic preconditioning (HP) and pharmacological preconditioning, using diazoxide (30 μM) or nicorandil (100 μM), also increased the time taken to induce the mPT by 2.0-, 2.1-, and 1.5-fold, respectively (P < 0.001), and rigor contracture by 1.9-, 1.7-, and 1.5-fold, respectively, compared with control (P < 0.001). Effects of HP, diazoxide, and nicorandil were abolished in the presence of mitochondrial ATP-sensitive K⁺ (KATP) channel blockers glibenclamide (10 μM) and 5-hydroxydecanoate (100 μM) but were maintained in the presence of the sarcolemmal K⁺ channel blocker HMR-1098 (10 μM). In conclusion, preconditioning protects the myocardium by reducing the probability of the mPT, which normally occurs during ischemia-reperfusion in response to oxidative stress.

ISCHEMIC PRECONDITIONING (IPC) confers profound protection on the myocardium in response to ischemia-reperfusion injury and refers to the phenomenon whereby one or more brief periods of sublethal ischemia render the myocardium more resistant to a subsequent more prolonged lethal ischemic insult (33). Despite intensive investigation, the actual mechanism of protection remains elusive. Studies have shown that the protective effect can be mimicked by certain pharmacological agents (so-called pharmacological preconditioning), such as diazoxide and pinacidil, that are purported to open the mitochondrial ATP-sensitive K⁺ (mKATP) channel (15), although the actual identity of the mKATP channel is unknown. Most of the evidence supporting its role in preconditioning has been obtained through the pharmacological manipulation of the channel, using agents such as diazoxide and pinacidil. However, both these drugs have also been shown to exert effects on mitochondrial respiratory function, which may explain their cardioprotective properties without evoking activation of the mKATP channel (17, 35).

Several studies have shown consequences of IPC and pharmacological preconditioning that could be beneficial for mitochondrial function and cell survival in the setting of ischemia-reperfusion. These consequences include 1) reducing mitochondrial calcium load (19, 32, 43); 2) attenuation of the oxidative stress generated at reperfusion (36, 42); 3) improved mitochondrial respiratory function (14, 24); 4) maintaining the integrity of the mitochondrial intermembrane space, which has important implications for mitochondrial respiratory function (8, 29); and 5) reducing mitochondrial cytochrome c release and apoptosis (1). More recently, it has been suggested that the preconditioning stimulus may act as a “trigger” for other downstream effector mechanisms that initiate myocardial preconditioning, with reactive oxygen species (ROS) acting as the mediator, although the end effector of preconditioning remains elusive (3, 13, 37).

A potential target in this scheme may be the mitochondrial permeability transition (mPT), which represents a fundamental event in the pathway to reperfusion-induced cell death (6). The mPT has been shown to be mediated by the opening of a nonspecific large conductance pore of the inner mitochondrial membrane (20). Inducing the mPT permeates the inner mitochondrial membrane, leading to cell death by apoptosis (due to the release of mitochondrial cytochrome c) and necrosis (due to the collapse of the mitochondrial membrane potential and resultant uncoupling of oxidative phosphorylation, which leads to ATP depletion) (30).

Interestingly, the probability of inducing the mPT is increased by the same factors that prevail in the setting of ischemia-reperfusion injury and include oxidative stress, a high mitochondrial calcium and inorganic phosphate load, and ATP depletion (4). Therefore, conditions for inducing the mPT are present during ischemia-reperfusion, and its role in mediating the cell death associated with ischemia-reperfusion injury has been borne out by the fact that we and others have shown that pharmacologically suppressing the mPT in this setting is cardioprotective (16, 18). Because preconditioning has been shown also to modulate factors associated with inducing the mPT, one might expect it to influence the probability of inducing the mPT in the setting of ischemia-reperfusion injury.

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We propose that preconditioning protects the myocardium by suppressing the mPT that occurs on reperfusing ischemic myocardium. In this regard, we have recently demonstrated for the first time the modulation of the mPT in the setting of IPC and pharmacological preconditioning (18). We investigated the effect of preconditioning on the mPT indirectly in the isolated perfused heart, and in isolated mitochondria we examined modulation of the mPT directly in the setting of pharmacological preconditioning (18). The objective of the present study was therefore to investigate more directly, using the intact cell, whether preconditioning protects by suppressing the mPT. Because ROS generated within mitochondria on reperfusing ischemic myocardium have been shown to play a pivotal role in both mediating cell death and inducing the mPT (28, 48), we investigated the effects of hypoxic and pharmacological preconditioning using a model in which oxidative stress generated within mitochondria is used to provoke the mPT followed by rigor contracture.

EXPERIMENTAL PROCEDURES

**Animals.** Male Sprague-Dawley rats (300 ± 50 g body wt) were obtained from Charles River UK Limited (Margate, UK) and received humane care in accordance with The Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (The Stationery Office, London, UK).

**Materials.** Diazoxide (Sigma) and glibenclamide (Sigma) were dissolved in DMSO, giving a final concentration of <0.1% DMSO. Cyclosporin A (CsA; Sigma) and N-methyl-4-valine-CsA (gift from Novartis Pharm; Basel, Switzerland) were dissolved in 50% ethanol, giving a final concentration of 0.05% ethanol. HMR-1098 (gift from Dr. J. Downey) and 5-hydroxydecanoate (5-HD; Sigma) were dissolved in distilled water. Tetramethylrhodamine methyl ester (TMRM; Molecular Probes Europe, Leiden, The Netherlands) was dissolved in DMSO. All other agents were of standard analytical grade and quality.

**Preparation of adult rat myocytes.** Adult rat myocytes were isolated by collagenase perfusion by use of a previously described method with modifications (45). Briefly, after anesthesia with sodium pentobarbital (55 mg/kg ip) and administration of heparin sodium (300 IU), hearts were rapidly excised, placed in ice-cold buffer, and mounted on a nonrecirculating perfusion apparatus. All solutions used were based on a modified calcium-free Krebs-Ringer-HEPES (KRH) buffer (in mM): 116.0 NaCl, 5.4 KCl, 0.4 MgSO4, 20.0 HEPES, 0.9 Na2HPO4, and 5.6 glucose (pH 7.4). The perfusate was bubbled with 100% O2 and maintained at 37°C. The hearts were first perfused at 14 ml/min with KRH buffer containing 1 mg/ml BSA and 3.3 μM EGTA. After 5 min, the hearts were perfused with KRH buffer containing 0.75 mg/ml collagenase (Worthington type II) and 25 μM calcium for 10–15 min. They were finally perfused with KRH buffer containing 50 μM calcium for 5 min. After perfusion, the hearts were removed from the perfusion apparatus, and the atria were trimmed away. The ventricles were minced and underwent several more digestions with collagenase. The cells were then filtered through a nylon mesh and washed with restoration buffer: KRH buffer plus 10 mg/ml BSA, 0.5 mM Na-pyruvate, 5.0 mM taurine, 2.0 mM carnitine, 1.0 mM creatine, and 75 μM calcium. The calcium concentration was gradually increased to 1.25 mM. After isolation, the cells were seeded onto sterilized laminin-coated 25-mm-diameter round coverslips and incubated overnight at 37°C in an atmosphere of 95% air-5% CO2 in M-199 medium (M7653, Sigma) containing 10% fetal calf serum and 1% penicillin-streptomycin (Sigma). The next day, cells were washed and kept in restoration buffer.

**Model for induction and detection of the mPT in intact cells.** We monitored induction of the mPT in a cellular model of oxidative stress (25). Isolated adult ventricular myocytes, in restoration buffer, were incubated with the fluorescent dye TMRM (3 μM) for 15 min at 37°C, washed, and visualized using confocal fluorescence microscopy. TMRM, a lipophilic cation, accumulates selectively into mitochondria according to the mitochondrial membrane potential. Laser illumination of the TMRM generates ROS from within the mitochondria that provoke the mPT. In this model, induction of the mPT is seen by collapse of the mitochondrial membrane potential (9, 22). Oxidative stress generated on reperfusing ischemic myocardium also involves a loss of mitochondrial membrane depolarization results in loss of dye into the cytosol where the signal increases (2, 10). Laser-induced oxidative stress was applied until the mPT had been provoked (indicated by collapse of the mitochondrial potential) and continued until rigor contracture had been induced. The time taken to induce the mPT and the additional time required to induce rigor contracture (signaling ATP depletion) were measured.

To confirm the efficacy of the protocol in inducing the mPT, we also examined the effect of laser-induced oxidative stress on the redistribution of the fluorescent dye calcein from the mitochondrial matrix. We used an established method for detecting the induction of the mPT in the intact cell (26, 38) in which adult rat myocytes were incubated with calcein-AM (1.0 μmol/l) and cobalt chloride (CoCl2, 1.0 mmol/l). The cobalt quenches cytosolic calcein so that only the mitochondrial dye is seen. In this model, the mPT is indicated by the redistribution of the calcine signal out of the mitochondrial matrix. This was quantified by measuring the ratio of the standard deviation to the mean of the fluorescence signal. Localization of the signal to mitochondria results in a signal in which pixels over mitochondria are very bright and pixels in the cytosol are very dark (SD is high). Redistribution of dye will lead to a decrease in mean signal as the calcein is quenched but also a loss of significance as the distribution of signal becomes increasingly uniform.

**Confocal fluorescence imaging and analysis.** The coverslip containing the myocytes was placed in a chamber and mounted on the stage of a Zeiss 510 CLSM confocal microscope equipped with ×40 oil immersion, quartz objective lens (numerical aperture 1.3). For TMRM fluorescence, the cells were illuminated by use of the 543-nm emission line of a HeNe laser. For all photosensitization experiments, all conditions of the confocal imaging system (laser power; confocal pinhole, set to give an optical slice of 1 μm; pixel dwell time; and detector sensitivity) were identical to ensure comparability between experiments. The fluorescence of TMRM was collected using a 585-nm long-pass filter. Images were analyzed by use of Zeiss software (LSM 2.8). For measurement of calcein fluorescence, cells were illuminated using the 488-nm emission line of an Argon laser, and fluorescence was measured by use of a 505-nm long-pass filter.

**Treatment protocols.** After being loaded with TMRM, cells were randomly assigned to the following treatment groups.

**Treatment group 1** is control (n = 18) or incubation with 0.05% ethanol vehicle (n = 6) or 0.1% DMSO vehicle (n = 6).

**Treatment groups 2 and 3** are CsA (0.2 μM, a known inhibitor of the mPT; n = 12) (5) and N-methyl-4-valine-CsA (0.4 μM, a known inhibitor of the mPT that does not inhibit the phosphatase calcineurin; n = 12), respectively (39).

**Treatment group 4** is hypoxic preconditioning (HP; n = 12). Cells were incubated for two periods of 10 min at 37°C in anoxia buffer composed of (in mM) 137.0 NaCl, 12.0 KCl, 0.49 MgCl2, 4.0 HEPES, 0.9 CaCl2, 1.0 Na-dithionite, 20.0 2-deoxyglucose, and 20.0 lactate.
(pH 6.5) with an intervening 30-min reoxygenation in restoration buffer before undergoing the TMRM-induced oxidative stress protocol.

Treatment groups 5 and 6 are HP in the presence of either 5-HD (100 μM, a purported mK<sub>ATP</sub> channel blocker; n = 6) or glibenclamide (10 μM, a nonspecific mK<sub>ATP</sub> channel blocker; n = 6), respectively.

Treatment group 7 is diazoxide (30 μM, a purported mK<sub>ATP</sub> channel opener; n = 6) (15).

Treatment groups 8, 9, and 10 are diazoxide in the presence of either 5-HD (n = 6), glibenclamide (n = 6), or HMR-1098 (10 μM, a specific sarcolemmal K<sub>ATP</sub> channel blocker; n = 6), respectively.

Treatment group 11 is nicorandil (100 μM, another purported mK<sub>ATP</sub> channel opener; n = 12).

Treatment groups 12 and 13 are nicorandil in the presence of either 5-HD (n = 6) or glibenclamide (n = 6), respectively.

Treatment groups 14, 15, and 16 are 5-HD (n = 6), glibenclamide (n = 6), or HMR-1098 alone (n = 6), respectively.

In all groups, the cells were incubated for 20 min with the treatment drug(s) before the TMRM-induced oxidative stress protocol.

Statistical analysis. All values are expressed as means ± SE. Times taken to induce global mitochondrial depolarization and rigor contracture were analyzed by one-way ANOVA and Fisher’s protected least significant difference test for multiple comparisons. Differences were considered significant when P < 0.05.

RESULTS

Model for induction and detection of the mPT in intact cells. Confocal fluorescence imaging of adult rat ventricular myocytes loaded with TMRM revealed mitochondria as fluorescent bands orientated with the longitudinal axis of the cell (Fig. 1A).
TMRM localizes selectively to the mitochondria according to the mitochondrial membrane potential. Figure 1 (A–E) shows representative images extracted from a time sequence in which a myocyte was loaded with TMRM and subjected to laser-induced oxidative stress and demonstrates the sequential changes that take place in mitochondrial membrane potential over time. Laser illumination first induces occasional and reversible local depolarizations of individual mitochondria (which appear as areas in which TMRM fluorescence suddenly disappears; Fig. 1B, arrows). With continued laser-induced oxidative stress, global mitochondrial membrane depolarization occurs, seen usually as a wave of increased TMRM fluorescence that propagates slowly and progressively from one end of the cell to the other, reflecting progression of irreversible mPT pore opening (Fig. 1C, arrow, and Fig. 2, A and B). The basis for the propagation of this wave of global mitochondrial depolarization is not known but may represent a self-propagating wave of mitochondria undergoing the mPT, with calcium and/or ROS released from one mitochondria on induction of the mPT, initiating the mPT in the adjacent mitochondria and so on (23, 40), until the whole cell has undergone global mitochondrial depolarization (Fig. 1D). After the depolarization, mitochondria consume ATP, ultimately leading to rigor contracture (Fig. 1E). The times taken to induce global mitochondrial membrane depolarization and rigor contracture were noted, and in the control group, the mPT was routinely induced after 252.0 ± 18.1 s of laser-induced oxidative stress. A further 384 s were required for the progression to irreversible contraction, with rigor contracture occurring after a total of 636.1 ± 26.4 s of laser-induced oxidative stress.

To verify that the observed global mitochondrial membrane depolarization induced by laser-induced oxidative stress represents the mPT, it is necessary to demonstrate that it is sensitive to CsA, the most reliable inhibitor of the mPT (5). CsA was shown to extend the time required to induce the mPT more than twofold, from 252.0 ± 18.1 to 450.7 ± 26.1 s, and it also extended the time required to induce rigor contracture from 636.1 ± 26.4 to 945.4 ± 69.7 s (P < 0.001; Figs. 3 and 4). However, as well as inhibiting the mPT, CsA inhibits the phosphatase calcineurin. To exclude any effect of CsA-induced inhibition of calcineurin in this model, we tested the nonimmunosuppressive CsA analog, N-methyl-4-valine-CsA, as this drug has been shown to inhibit the mPT without inhibiting calcineurin (39). This mPT inhibitor also increased the time taken to induce both mPT and rigor contracture to 605.8 ± 74.5 s and 1055.3 ± 79.4 s, respectively (P < 0.001; Figs. 3 and 4). Furthermore, in this model, N-methyl-4-valine-CsA was shown to be a more potent inhibitor of the mPT than CsA, extending the time taken to induce the mPT even further than CsA (605.8 ± 74.5 s with N-methyl-4-valine-CsA vs. 450.7 ± 26.1 s with CsA; P < 0.005; Fig. 3), although there was no significant difference with respect to the times taken to induce rigor contracture. One can speculate that its greater specificity for inhibiting the mPT than CsA may explain its more potent inhibition of the mPT (39).

Laser irradiation of myocytes loaded with calcein (in the presence of cobalt) resulted in the redistribution of calcein fluorescence out of the mitochondrial matrix, indicating...
induction of the mPT (Fig. 5; n = 4), confirming that laser-induced oxidative stress is sufficient in itself to induce the mPT in adult rat myocytes, and supporting the interpretation of phototoxicity-induced mitochondrial depolarization as a reflection of mPT.

**Hypoxic and pharmacological preconditioning protects myocytes from oxidative stress by inhibiting the mPT.** Pretreatment of cells by either hypoxic or pharmacological preconditioning, using diazoxide or nicorandil, also increased the illumination time required to induce global mitochondrial membrane depolarization from 252.0 ± 18.1 s in control to 464.1 ± 53.8 s in hypoxic preconditioned cells, 528.5 ± 28.5 s after diazoxide, and 386.1 ± 33.4 s after nicorandil (P < 0.001; Figs. 3 and 6). The times taken to induce rigor were also extended from 636.1 ± 26.4 s in control to 1,140.7 ± 84.0 s, 1,095.6 ± 102.4 s, and 967.6 ± 61.8 s, respectively (P < 0.001; Figs. 4 and 7). These results suggest that hypoxic and pharmacological preconditioning all act to inhibit the mPT induced by oxidative stress, resulting in protection from oxidative stress-induced rigor contracture.

The purported mK<sub>ATP</sub> channel blockers glibenclamide and 5-HD abrogated this effect of hypoxic and pharmacological preconditioning on the mPT and ATP depletion. HP in the presence of either glibenclamide or 5-HD abolished both the delay in the time taken to induce the mPT (464.1 ± 53.8 s with HP vs. 256.6 ± 27.0 s with glibenclamide and 247.4 ± 7.0 s with 5-HD; P < 0.001; Fig. 3) and the delay in the time taken to induce rigor contracture (1,140.7 ± 84.0 s with HP vs. 692.3 ± 68.0 s with glibenclamide and 687.8 ± 30.1 s with 5-HD; P < 0.001; Fig. 4).

With respect to pharmacological preconditioning, diazoxide in the presence of either glibenclamide or 5-HD also abolished both the delay in the time taken to induce the mPT (528.3 ± 28.5 s with diazoxide vs. 261.7 ± 24.1 s with glibenclamide and 283.3 ± 14.4 s with 5-HD; P < 0.001; Fig. 6) and the delay in the time taken to induce rigor contracture (1,095.6 ± 102.4 s with diazoxide vs. 543.4 ± 47.1 s with glibenclamide and 664.0 ± 48.3 s with 5-HD; P < 0.001; Fig. 7). Furthermore, nicorandil in the presence of either glibenclamide or 5-HD also abolished both the delay in the time taken to induce the mPT (386.1 ± 33.4 s with nicorandil vs. 256.1 ± 25.6 s with glibenclamide and 249.1 ± 36.1 s with 5-HD; P < 0.001; Fig. 6) and the delay in the time taken to induce rigor contracture (967.6 ± 61.8 s with nicorandil vs. 482.3 ± 48.1 s with glibenclamide and 661.0 ± 77.4 s with 5-HD; P < 0.001; Fig. 7).

However, the specific sarcolemmal K<sub>ATP</sub> channel blocker HMR-1098 did not abolish the effects of diazoxide on either the time taken to induce the mPT [528.5 ± 28.5 s with diazoxide vs. 485.9 ± 11.5 s with HMR-1098; P = not significant (NS); Fig. 6] or the time taken to induce rigor contracture (1,095.6 ± 102.4 s with diazoxide vs. 1,076.8 ± 30.3 s with HMR-1098; P = NS; Fig. 7). Given alone, the K<sub>ATP</sub> channel blockers did not influence either the time required to induce the mPT (216.3 ± 13.5 s in control vs. 239.4 ± 22.3 s with glibenclamide, 197.3 ± 11.2 s with 5-HD, and 216.3 ± 13.5 s with HMR-1098; P = NS; Figs. 3 and 6) or the time taken to induce rigor contracture (636.1 ± 26.4 s in control vs. 732.6 ± 77.3 s with glibenclamide, 496.4 ± 28.2 s with 5-HD, and 729.4 ± 18.8 s with HMR-1098; P = NS; Figs. 4 and 7).

**DISCUSSION**

This study shows for the first time that hypoxic and pharmacological preconditionings protect the myocyte by suppress-
ing the mPT induced in the setting of oxidative stress. Myo-
cytes treated by preconditioning were shown to be more
resistant to oxidative stress, and the time taken to induce rigor
contracture was shown to relate directly to suppression of the
mPT. We chose to examine the induction of the mPT in the
setting of oxidative stress, as this relationship pertains to events
that occur in ischemia-reperfusion. Reperfusion of ischemic
myocardium has been shown not only to generate oxidative
stress but also to induce the mPT, both factors that contribute
to the cell death associated with ischemia-reperfusion
(28, 48). We used a well-established model for inducing and
detecting the mPT in the intact cell (7, 9, 11, 21, 22, 25, 47).
Interestingly, we show that a preconditioning stimulus is able
to protect by modulating crucial events that take place at
reperfusion that determine cell survival in the setting of ische-
mia-reperfusion. Specifically, both hypoxic and pharmacolog-
ical preconditioning were shown to inhibit the mPT that nor-
mally occurs in response to oxidative stress, a major determi-
nant of reperfusion-induced cell death.

The present study extends previous work we have under-
taken (18) in which we showed for the first time that cardio-
protection arising from either ischemic or pharmacological
preconditioning may be due to suppression of the mPT that
normally occurs on reperfusing ischemic myocardium. In that
study, we demonstrated in the isolated perfused rat heart that
pharmacologically inducing the mPT at reperfusion reversed
the protection induced by both ischemic and pharmacological
preconditioning, using infarct size as the measured end point of
cell death. This finding suggested to us that preconditioning
may manifest its protection by suppressing the mPT but did not
directly prove it. To prove suppression of the mPT as the
mechanism responsible for preconditioning-induced protec-
tion, it was imperative to assess the consequences of precon-
ditioning directly. In this regard, we and others (18, 27) have shown that diazoxide, an agent used to pharma-
cologically precondition the myocardium, was able to inhibit
the mPT associated with mitochondrial calcium loading, a
major contributor to reperfusion-induced cell death. An inter-
esting study by Xu et al. (46) in myocytes had previously
shown that pharmacologically inducing the mPT abrogated
the protection associated with calcium-induced preconditioning.
However, the authors failed to examine the consequence of

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**Fig. 6.** Effect of pharmacological preconditioning using
diazoxide (DZX) or nicorandil (NIC) in the presence or
absence of the mK_ATP channel blockers 5-HD, Glib, and
HMR-1098 (HMR) on the time taken to induce the mPT in
TMRM-loaded myocytes. Values are means ± SE; n =
6/group. *P < 0.001.

**Fig. 7.** Effect of pharmacological preconditioning using
DZX or NIC in the presence or absence of the mK_ATP
channel blockers 5-HD, Glib, and HMR on the time taken to
induce rigor contracture in TMRM-loaded myocytes. Values
are means ± SE; n = 6/group. *P < 0.001.
calcium-induced preconditioning on the mPT directly. In the present study, we examined directly the effect of hypoxic and pharmacological preconditioning on the mPT in the intact cell.

The effects of hypoxic and pharmacological preconditioning (using diazoxide or nicorandil, purported openers of the mKATP channel) on suppressing the mPT and protecting against rigor contracture in the face of oxidative stress were abrogated in the presence of 5-HD and glibenclamide, agents that have been shown to antagonize the putative mKATP channel. Controversy surrounds the nature of protection associated with agents such as diazoxide and pinacidil that are reported to act via the putative mKATP channel (17, 35). Methods employed in investigating the role of the mKATP channel have relied heavily on using pharmacological agents to manipulate the mKATP channel (15, 31). It is clear that agents such as diazoxide or pinacidil do protect the myocardium from ischemia-reperfusion injury, but because they also have nonspecific effects on mitochondrial function, the mechanism through which they cardioprotect may be independent of mKATP channel activation. For example, as far back as 1969, it was noted that diazoxide can inhibit the electron transport carrier II, succinate dehydrogenase (SDH) (41). Several studies have confirmed this effect of diazoxide on SDH and have shown that this effect is not restricted to diazoxide and may also apply to pinacidil (17, 35). Even abrogation by 5-HD of the cardioprotective effect induced by diazoxide or pinacidil may be explained by a nonspecific effect of 5-HD on mitochondrial function (17). Recently, it has been shown that 5-HD can be metabolized to a fatty acid that acts as a substrate for SDH, thereby antagonizing the inhibitory effects of diazoxide or pinacidil on SDH rather than antagonizing at the mKATP channel (17). Furthermore, it has been demonstrated that inhibition of SDH, using a low dose of 3-nitropropionic acid (3-NPA), protected rabbit hearts from infarction, an effect abolished by 5-HD (34).

By inhibiting electron flow through the electron transport chain, agents such as diazoxide or pinacidil may be expected to generate ROS from complexes I or III (12). It is exciting to consider that this may be the source for the ROS that have been recently implicated as mediating preconditioning-induced protection through their activation of prosurvival kinases. (3, 13, 37). In this regard, it has been shown that in cerebral tissue,

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AT REPERFUSION
mPTP OPENS

- Water and solutes
- Apoptosis
- Ruptured membrane
- Cytochrome C
- Cyclophilin D

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ISCHEMIC PRECONDITIONING

AT REPERFUSION
mPTP CLOSED

- \( K_{\text{ATP}} \) channel
- VDAC
- ANT
- Cytochrome C
- Cyclophilin D

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1. Reduction in matrix \( \text{Ca}^{2+} \) loading
2. Improved energy production
3. Decreased release of ROS at time of reperfusion

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mPTP remains closed at reperfusion due to reduced [\( \text{Ca}^{2+} \)] load, maintained ATP levels, and less oxidative stress.

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**Fig. 8.** A: scheme showing how opening of the mPTP at the time of reperfusion mediates cell death. Opening of the mPTP, which occurs in response to reactive oxygen species (ROS) and calcium, permeates the inner mitochondrial membrane; this allows water to enter the mitochondrial matrix, which leads to rupture of the outer mitochondrial membrane, thereby allowing the release of cytochrome c and other proapoptotic factors into the cytosol. The mPTP is believed to be composed of 3 core components: the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane, the adenine nucleotide translocase (ANT) of the inner mitochondrial membrane, and mitochondrial cyclophilin D. B: proposed scheme depicting how mPTP opening may be inhibited at the time of reperfusion in response to ischemic preconditioning and mitochondrial \( K_{\text{ATP}} \) channel opening. Preconditioning confers beneficial effects on mitochondrial function that act in concert to reduce the opening probability of the mPTP opening at the time of reperfusion, thereby mediating cellular protection.
3-NPA, through its inhibition of SDH, caused a burst of ROS, which were then shown to mediate preconditioning-type protection (44).

It may transpire therefore that ischemic and pharmacological preconditionings (using agents such as diazoxide and nicorandil) induce cardioprotection through their nonspecific effects on mitochondrial function, including respiratory chain inhibition, and ROS release from the mitochondria. We propose that preconditioning confers beneficial effects on mitochondrial function and calcium handling that result in mitochondria that are better equipped or “preconditioned” to withstand the crucial phase of reperfusion, which is associated with an influx of calcium into the mitochondria and oxidative stress (see Fig. 8). Although these events would normally precipitate the mPT and cell death, the events of preconditioning reduce the probability of inducing the mPT at reperfusion and so protect the heart from cell death.

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