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Transient Mitochondrial Permeability Transition Pore Opening Mediates Preconditioning-Induced Protection

Derek Hausenloy, MBChB; Abigail Wynne, BSc; Michael Duchen, PhD; Derek Yellon, DSc

Background—Transient (low-conductance) opening of the mitochondrial permeability transition pore (mPTP) may limit mitochondrial calcium load and mediate mitochondrial reactive oxygen species (ROS) signaling. We hypothesize that transient mPTP opening and ROS mediate the protection associated with myocardial preconditioning and mitochondrial uncoupling.

Methods and Results—Isolated perfused rat hearts were subjected to 35 minutes of ischemia/120 minutes of reperfusion, and the infarct-risk-volume ratio was determined by tetrazolium staining. Inhibiting mPTP opening during the preconditioning phase with cyclosporine-A (CsA, 0.2 μmol/L) or sanglifehrin-A (SfA, 1.0 μmol/L) abolished the protection associated with ischemic preconditioning (IPC) (20.2±3.6% versus 45.9±2.5% with CsA, 49.0±7.1% with SfA; $P<0.001$); and pharmacological preconditioning with diazoxide (Dzx, 30 μmol/L) (22.1±2.7% versus 46.3±3.0% with CsA, 48.4±5.5% with SfA; $P<0.001$), CCPA (the adenosine A1-receptor agonist, 200 nmol/L) (24.9±4.5% versus 54.4±6.6% with CsA, 42.6±9.0% with SfA; $P<0.001$), or 2,4-dinitrophenol (DNP, the mitochondrial uncoupler, 50 μmol/L) (15.7±2.7% versus 40.8±5.5% with CsA, 34.3±3.1% with SfA; $P<0.001$), suggesting that mPTP opening during the preconditioning phase is required to mediate protection in these settings. Inhibiting ROS during the preconditioning protocols with *N*-mercaptopropionylglycine (MPG, 1 mmol/L) also abolished the protection associated with IPC (20.2±3.6% versus 47.1±3.8% with MPG; $P<0.001$), diazoxide (22.1±2.7% versus 56.3±3.8% with MPG; $P<0.001$), and DNP (15.7±2.7% versus 50.7±6.6% with MPG; $P<0.001$) but not CCPA (24.9±4.5% versus 26.5±8.4% with MPG; P=NS). Further experiments in adult rat myocytes demonstrated that diazoxide induced CsA-sensitive, low-conductance transient mPTP opening (represented by a 28±3% reduction in mitochondrial calcein fluorescence compared with control; $P<0.01$).

Conclusions—We report that the protection associated with IPC, diazoxide, and mitochondrial uncoupling requires transient mPTP opening and ROS. **(*Circulation*. 2004;109:1714-1717.)**

Key Words: ischemia ■ myocardial infarction ■ free radicals ■ reperfusion

Despite ongoing intensive investigation, the actual mechanism responsible for the powerful protective phenomenon that is ischemic preconditioning (IPC)[1] has not yet been elucidated. Studies have implicated mitochondria in protective mechanisms associated with IPC. Pharmacological opening of the purported mitochondrial K_{ATP} channel[2] has been demonstrated to cardioprotect by preserving mitochondrial energy production during ischemia-reperfusion.[3] We and others have implicated modest mitochondrial uncoupling as a critical event in preconditioning-induced protection.[4,5] Mitochondrial reactive oxygen species (ROS) release may mediate the preconditioning signal.[6] We have demonstrated that the prolonged (high-conductance) mitochondrial permeability transition pore (mPTP) opening, which mediates cell death at the time of reperfusion, can be inhibited by preconditioning.[7]

The present study focuses on the physiological transient (low-conductance) form of mPTP opening, which does not lead to cell death[8] and in fact may play several important functions that may contribute to IPC-induced protection. Transient (low-conductance) mPTP opening (1) can limit mitochondrial matrix calcium load by mediating mitochondrial calcium efflux[9]; (2) can be induced by mitochondrial uncoupling[10]; and (3) can mediate mitochondrial ROS release.[11]

This would suggest that low-conductance transient mPTP opening may contribute to the mechanism of IPC-induced protection. In this regard, the preconditioning mimetic diazoxide has been demonstrated to induce mPTP opening.[12] On this basis, we hypothesized that both transient (low-conductance) mPTP opening and ROS, during the preconditioning phase, mediate both preconditioning and mitochondrial uncoupling–induced protection.

Methods

Isolated Perfused Rat Heart

Hearts excised from male Sprague-Dawley rats were Langendorff-perfused with Krebs-Henseleit buffer and subjected to 35 minutes of

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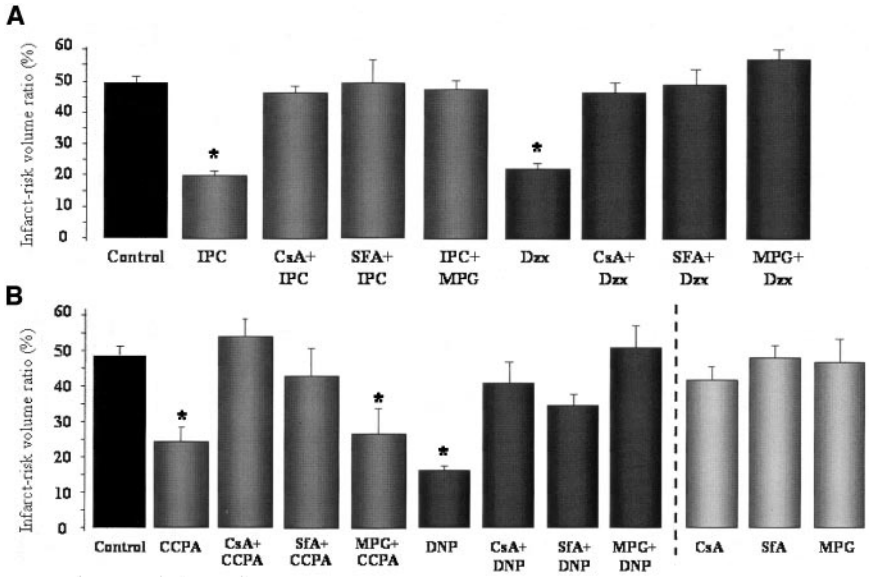

Figure 1. A, Inhibiting mPTP opening with either CsA or SfA or scavenging ROS by using MPG during preconditioning phase abolishes protection associated with IPC and Dzx. B, Inhibiting mPTP opening with either CsA or SfA during preconditioning phase abolishes protection associated with CCPA and DNP. Scavenging ROS during preconditioning phase by using MPG abolishes protection associated with DNP but not CCPA (*$P<0.05$).

ischemia followed by 120 minutes of reperfusion, and the infarct-risk-volume ratio was determined by triphenyltetrazolium-chloride staining.[7]

Treatment Protocols for Infarct Studies

The hearts were treated as follows (n≥6/group):

(1) Control hearts were perfused with 0.02% DMSO or 0.005% ethanol, or Krebs-Henseleit buffer alone during stabilization.
(2) IPC hearts were treated with two 5-minute periods of global ischemia with a 10-minute intervening reperfusion.
(3, 4) Hearts underwent the IPC protocol in the presence of the mPTP inhibitors cyclosporine-A (CsA 0.2 μmol/L, Sigma)[7] or sanglifehrin-A (SfA 1.0 μmol/L, Novartis).[13]
(5) Hearts underwent IPC in the presence of *N*-mercaptopropionylglycine (MPG, the ROS scavenger, 1 mmol/L).[14]
(6, 7) Hearts were perfused with diazoxide (Dzx, 30 μmol/L)[2] for 10 minutes or the adenosine A1-receptor agonist CCPA (200 nmol/L)[7] for 10 minutes (during which the hearts were paced at 300 bpm for CCPA-induced bradycardia) followed by 10 minutes of washout.
(8–13) Hearts were preconditioned with diazoxide or CCPA in the presence of CsA/SfA/MPG.
(14) Hearts were perfused with 2,4-dinitrophenol (DNP, a mitochondrial uncoupler, 50 μmol/L)[4] for 5 minutes followed by 10 minutes of washout.
(15–17) Hearts were perfused with DNP in the presence of CsA/SfA/MPG.
(17–19) Hearts were perfused with CsA/SfA/MPG during stabilization.

Model for Detecting mPTP Opening in Intact Cells

We used an established method for detecting transient (low-conductance) mPTP opening in the intact cell.[12,15] Adult rat myocytes isolated by collagenase perfusion from Sprague-Dawley rats, with the use of a previously described method,[16] were incubated with calcein-AM (1.0 μmol/L) and cobalt-chloride ($CoCl_2$, 1.0 mmol/L), resulting in mitochondrial localization of calcein fluorescence. mPTP opening was indicated by a reduction in mitochondrial calcein signal (expressed as the percentage of the baseline value) and was measured over 6 randomly chosen areas in 3 different cells every 5 minutes for 25 minutes, with the use of a Zeiss-510 CLSM confocal microscope (emitting at 488 nm and detecting at 505 nm).

Cells were incubated for 20 minutes at 37°C with (1) 0.05% ethanol vehicle (n=6); (2) 0.1% DMSO vehicle (n=6); or (3) diazoxide (n=18/group;30 μmol/L) in the presence or absence of CsA (0.2 μmol/L) or 5-HD (100 μmol/L).[2]

Statistical Analysis

All values are expressed as mean±SEM. Infarct-risk-volume ratios and mitochondrial calcein fluorescence intensities were analyzed by 1e-way ANOVA and Fisher's protected least significant difference test for multiple comparisons. Differences were considered significant at a value of $P<0.05$.

Results

Opening of the mPTP Is Required for Protection

Ischemic preconditioning, diazoxide, CCPA, or DNP reduced infarct size from 49.9±3.8% in control to 20.2±3.6% with IPC, 22.1±2.7% with diazoxide, 24.9±4.5% with CCPA, and 15.7±2.7% with DNP ($P<0.001$; Figure 1A). Inhibiting mPTP opening during the preconditioning protocol, with the use of CsA/SfA, abolished the protection associated with IPC (20.2±3.6% versus 45.9±2.5% with CsA, 49.0±7.1% with SfA; $P<0.001$; Figure 1A), diazoxide (22.1±2.7% versus 46.3±3.0% with CsA, 48.4±5.5% with SfA; $P<0.001$; Figure 1A), CCPA (24.9±4.5% versus 54.4±6.6% with CsA, 42.6±9.0% with SfA; $P<0.001$; Figure 1B), and DNP (15.7±2.7% versus 40.8±5.5% with CsA, 34.3±3.1% with SfA; $P<0.001$; Figure 1B), suggesting that mPTP opening is required to mediate the protection in these settings. Given alone, neither cyclosporine-A nor sanglifehrin-A influenced infarct size (43.9±1.4% in control versus 42.8±3.5% with CsA, 48.0±4.2% with SfA; P=NS; Figure 1B).

Reactive Oxygen Species Are Required for Protection

The presence of the ROS scavenger MPG during the preconditioning protocols abolished the protection associated with IPC (20.2±3.6% versus 47.1±3.8% with MPG; $P<0.001$; Figure 1A), diazoxide (22.1±2.7% versus 56.3±3.8% with MPG; $P<0.001$; Figure 1A), and DNP (15.7±2.7% versus 50.7±6.6% with MPG; $P<0.001$; Figure 1B), implicating ROS as a mediator of protection in these settings. However,

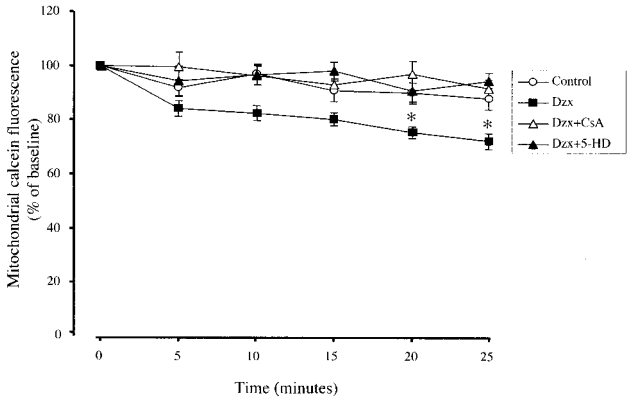

Figure 2. Mitochondrial calcein fluorescence (expressed as percentage of baseline fluorescence) in myocytes loaded with calcein and cobalt chloride demonstrate Dzx-induced reduction in mitochondrial calcein fluorescence, indicating transient (low-conductance) mPTP opening, which is abolished in the presence of either 5-HD (a mitochondrial K_{ATP} channel blocker) or CsA (an mPTP inhibitor) (*$P<0.05$).

MPG did not abolish the protection associated with CCPA (24.9±4.5% versus 26.5±8.4% with MPG; $P<0.001$; Figure 1B). MPG alone did not influence infarct size (43.9±1.4% in control versus 47.8±6.4% with MPG; P=NS; Figure 1B).

Diazoxide Induces Low-Conductance Transient mPTP Opening

Treatment of calcein-loaded myocytes with diazoxide resulted in a reduction in mitochondrial calcein fluorescence to 72±3% of baseline values ($P<0.01$; Figure 2), indicating that diazoxide induces low-conductance transient mPTP opening in quiescent cells. This effect of diazoxide was abolished by cyclosporine-A (the mPTP inhibitor, confirming that the reduction in mitochondrial calcein fluorescence was due to mPTP opening) and by 5-HD (the mitochondrial K_{ATP} channel blocker) (Figure 2).

Discussion

We report that transient (low-conductance) mPTP opening and ROS, during the preconditioning phase, are required to mediate the protection associated with ischemic and pharmacological preconditioning and mitochondrial -uncoupling. In the infarct studies, we demonstrated that pharmacologically inhibiting mPTP opening during the preconditioning phase completely abrogated the protection associated with IPC, diazoxide, and CCPA, indicating that mPTP opening is required for protection in these settings. In the myocyte model of mPTP opening,[12,15] we demonstrated that diazoxide induces transient (low-conductance) mPTP opening, confirming the findings of previous studies.[12,17] We confirm that IPC and diazoxide-induced protection is ROS-dependent and found that CCPA-induced preconditioning is ROS-independent, supporting the findings of Cohen and colleagues.[6]

We have previously demonstrated that modest mitochondrial uncoupling is a critical event in preconditioning-induced protection.[4,5] In the present study, we show that this protection can be abolished by inhibiting mPTP opening, suggesting that mPTP opening occurs downstream of mitochondrial uncoupling, which is supported by the fact that mitochondrial uncoupling can induce mPTP opening.[10] Transient mPTP opening can induce mitochondrial ROS release,[11] which may explain why we found mitochondrial uncoupling–induced protection to be ROS-dependent.

Transient mPTP opening during the preconditioning phase may mediate protection by (Figure 3) reducing mitochondrial calcium load[9]: In this regard, diazoxide has been shown to induce mitochondrial calcium efflux through mPTP opening.[12] Transient mPTP opening during the preconditioning phase also may mediate protection by mediating mitochondrial ROS release/signaling[11]: We are undertaking further studies to determine whether preconditioning-induced mitochondrial ROS release occurs through mPTP opening.

Because transient mPTP opening can be induced by uncoupling, oxidation of NADH,[10] and an alkaline pH,[8] the

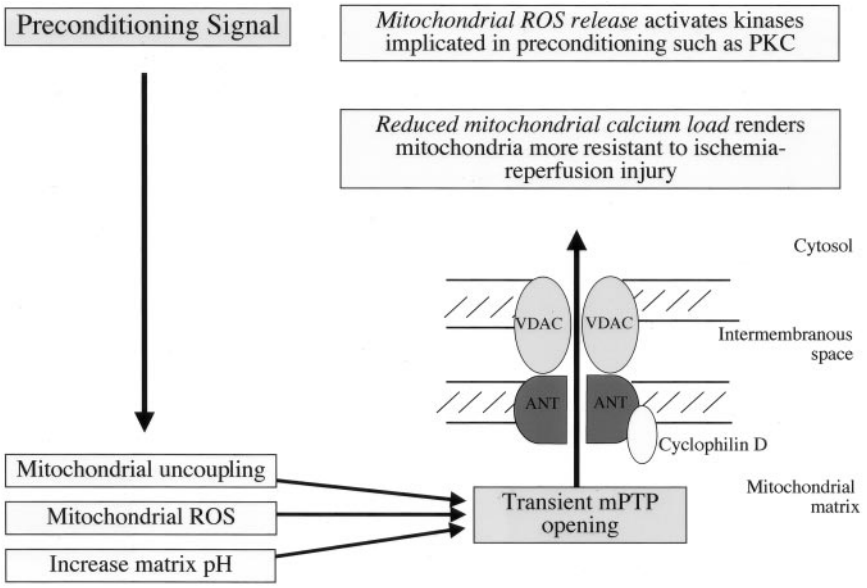

Figure 3. Hypothetical scheme outlining role for transient (low-conductance) mPTP opening in myocardial preconditioning. mPTP comprises the voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and cyclophilin D. Preconditioning induces transient mPTP (low-conductance) opening through mitochondrial uncoupling, ROS, and increasing matrix pH, which then protects the heart by reducing mitochondrial calcium load and facilitating ROS signaling.

preconditioning stimulus may induce transient mPTP opening by mediating uncoupling, producing mitochondrial ROS, which then oxidize NADH,[10] or by increasing matrix pH through activation of the mitochondrial K_{ATP} channel.[18]

In conclusion, we report for the first time that IPC, diazoxide, CCPA, and mitochondrial uncoupling all protect by inducing transient mPTP opening during the preconditioning phase. Given that the adenine nucleotide translocase (ANT) is believed to be a component of the mPTP[8] and the recent suggestion that the ANT forms part of the mitochondrial K_{ATP} channel,[19] it would be intriguing to speculate on whether agents that reportedly protect through opening of the mitochondrial K_{ATP} channel actually protect through transient (low-conductance) opening of the mPTP.

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