Ischemic and Pharmacological Preconditioning in Girardi Cells and C2C12 Myotubes Induce Mitochondrial Uncoupling

Jan Minners, Lydia Lacerda, Joy McCarthy, James J. Meiring, Derek M. Yellon, Michael N. Sack

Abstract—Pharmacological uncoupling of mitochondrial oxidation from phosphorylation promotes preconditioning-like cardioprotection in the isolated rat heart. We hypothesized that modest mitochondrial uncoupling may be a critical cellular event in orchestrating preconditioning. Human-derived Girardi cells and murine C2C12 skeletal myotubes were preconditioned using simulated ischemia, adenosine, and diazoxide. Cell viability after 6 hours of simulated ischemia was measured using lactate dehydrogenase release and propidium iodide uptake. Mitochondrial inner membrane potential (ΔΨm) was investigated by flow cytometry, cellular ATP by recombinant firefly-luciferase bioluminescence, and cellular oxygen consumption using oximetry. Preconditioning enhanced cell viability with attenuation of lactate dehydrogenase release (≥30%, P<0.05 versus ischemic controls) and a reduction in propidium iodide uptake by ≥26% versus ischemic controls after simulated ischemia in both cell lines. In Girardi cells, preconditioning induced the following phenotype immediately before index ischemia: (1) decreased ΔΨm (JC-1: simulated ischemia 90±3%, adenosine 82±7%, diazoxide 87±4%, versus control 100%, P<0.05); (2) attenuation in cellular ATP levels (CTL 0.21±0.03 nmol/L ATP/μg protein, simulated ischemia 0.12±0.02, adenosine 0.15±0.02, diazoxide 0.11±0.02, P<0.05); and (3) enhanced cellular oxygen consumption (control 2.3±0.1 nmol/L oxygen/min/1×10⁶ cells, simulated ischemia 3.1±0.1, adenosine 3.1±0.3, diazoxide 2.6±0.2, P<0.05). Cytoprotection, mitochondrial depolarization, and enhanced oxygen consumption were attenuated by the putative mitochondrial K_ATP-channel antagonist 5-hydroxydecanoate. The uncoupled phenotype in response to preconditioning was similarly observed in C2C12 myotubes. The present study suggests that modest mitochondrial uncoupling represents a unifying cellular response which may be important in directing preconditioning-mediated cytoprotection. (Circ Res. 2001;89:787-792.)

Key Words: preconditioning ■ mitochondrial function ■ mitochondrial membrane potential ■ ATP ■ oxygen consumption

Understanding the phenomenon of preconditioning may have widespread application in augmenting tissue tolerance against cell death following ischemia. The cellular survival program induced by preconditioning is still poorly understood. However, the intracellular signaling cascades activated by preconditioning triggers seem to converge on and activate/open the mitochondrial ATP-sensitive potassium channel (mK_ATP).1-3 The biological consequences of mK_ATP opening and its putative effects on enhancing tolerance to ischemia are less well-understood. Studies in isolated mitochondria suggest that mitochondrial matrix swelling, membrane depolarization, perturbation in ATP synthesis, and mitochondrial respiration are possible consequences of mK_ATP channel activation.3,4 These data are, however, being intensely debated, in part, because of the nonphysiological conditions wherein isolated mitochondrial studies are performed.5

We have previously demonstrated that transient mitochondrial uncoupling using 2,4-dinitrophenol (DNP) in the isolated perfused rat heart confers protection against myocardial infarction to a similar degree as ischemic preconditioning.6 Mitochondrial uncoupling describes a dissociation of mitochondrial respiration from ATP synthesis and is characterized by a dissipation of the H⁺ gradient across the inner mitochondrial membrane and a subsequent increase in oxygen consumption that is not associated with an increase in ATP production.7

To evaluate whether mitochondrial uncoupling is a universal component of the preconditioning program, we directly assessed whether a multitude of preconditioning triggers induce this phenotype. The mitochondrial phenotype was investigated in whole cells by measuring mitochondrial membrane potential (ΔΨm), oxygen consumption, and whole-cell ATP levels after the simulated ischemic, adenosine, and diazoxide preconditioning in two distinct transformed cell lines that had previously been studied in preconditioning.8,9

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After 1 hour of drug-free incubation, cells were either trypsinized and used for studies of mitochondrial function or underwent 6 hours of simulated ischemia with subsequent cell-viability measurements.

**C2C12 Myotube Preconditioning Protocols**

C2C12 myoblasts were obtained from the American Type Culture Collection. The rapidly dividing myoblasts were maintained in a 5% CO₂, DMEM, and 20% FBS until approximately 50% to 70% confluent. Differentiation to myotubes was initiated by changing to a 2% horse serum–supplemented DMEM and by maintaining the cells in an 8% CO₂ environment. Mature myotubes at 100% confluence were used for experiments between days 7 and 10. The preconditioning protocol was similar to that for Girardi cells, except that the preconditioning was extended from 30 minutes to 1 hour, and the index ischemic insult was extended to 8 hours.

**Measurement of Cell Viability**

Cell viability was evaluated by spectrophotometrical (Roche) measurement of lactate dehydrogenase (LDH) release into the medium and via the measurement of propidium iodide uptake on flow cytometry. Propidium iodide fluorescence was measured in a population of 1×10⁶ cells and was expressed as percent change over ischemic controls.

**Inner Mitochondrial Membrane Potential Analysis**

Cells were stained with 10 nmol/L DiOC₆₃ (3',3'-dihexyloxacarbocyanine iodide, Sigma) or JC-1 (5,5',6,6'-tetrachloro-1',1',3,3'-tetraethylrhodaminecarbocyanine, 1 µg/mL, Molecular Probes) for 30 minutes at 37°C, trypsinized, resuspended in medium at a density of approximately 10⁶/mL, and transferred on ice to the flow cytometer. DiOC₆ was excited at 488 nm and resultant fluorescence analyzed at 525 nm (FL1) on a FACSCalibur (Becton Dickinson) flow cytometer. JC-1 was excited at 488 nm and the monomer signal (green) was analyzed at 525 nm (FL1). Simultaneously, the aggregate signal (red) was analyzed at 590 nm (FL2). One minute before mitochondrial membrane potential measurement, propidium iodide (PI, 10 µg/mL) was added to exclude dead cells (FL3) from the analysis. In Girardi cells, the percentage of PI-positive cells was <1%, and <5% in C2C12 myotubes. PI-negative cells (1×10⁶) were analyzed and mean fluorescence was calculated for each sample.

**ATP Concentrations**

Cellular ATP concentration was measured by luciferin-luciferase luminometry. Protein concentrations were determined by the Lowry method and data are expressed as nanomoles ATP/µg protein.

**Oxygen Consumption**

Whole-cell respiration was measured using a Clark-type electrode with an integrated temperature control unit (Oxytherm). Oxygen consumption is expressed as nanomoles oxygen/min/1×10⁶ cells.

**Chemicals and Pharmacological Agents**

DiOC₆, adenosine, diazoxide, and 5-HD were obtained from Sigma. JC-1 was purchased from Molecular Probes. JC-1 and diazoxide were dissolved in DMSO. The final concentration of DMSO was <0.02%. DiOC₆ was dissolved in ethanol, adenosine, and 5-HD in distilled water. All pharmacological agents were stored at −20°C.

**Statistical Analysis**

Data are presented as mean±SEM. Comparisons between multiple groups were performed by one-way ANOVA followed by the Student-Newman-Keul post hoc test (Graph Pad Instat). A value of P<0.05 was considered statistically significant.
**Results**

**Preconditioning Improves Postischemic Cell Viability in Adherent Transformed Cell Lines**

Preconditioning Girardi cells reduced LDH release to $65 \pm 9\%$ (simulated ischemia), $68 \pm 4\%$ (adenosine), and $70 \pm 5\%$ (diazoxide) of nonpreconditioned control cells ($P<0.01$ versus controls, Figure 1B). Using propidium iodide exclusion as an index of cell viability, the corresponding values were $76 \pm 6\%$ (simulated ischemia), $72 \pm 4\%$ (adenosine), and $74 \pm 3\%$ (diazoxide, $P<0.01$ versus controls, Figure 1C).

Co-administration of 5-HD during preconditioning abolished the protection against a subsequent simulated ischemic insult. The LDH release after simulated preconditioning ischemia in the presence of 5-HD was $83 \pm 12\%$, adenosine + 5-HD was $92 \pm 4\%$, and after diazoxide + 5-HD, $102 \pm 5\%$ ($P=NS$ versus controls, Figure 1B). In parallel, propidium iodide uptake was increased to $107 \pm 9\%$ in cells undergoing simulated preconditioning ischemia + 5-HD, to $105 \pm 3\%$ in the adenosine + 5-HD, and to $98 \pm 5\%$ in the diazoxide + 5-HD group ($P=NS$ versus controls, Figure 1C). Similar cytoprotective effects of these preconditioning triggers were obtained using C2C12 myotubes (data not shown).

**Preconditioning Depolarizes Mitochondrial Inner Membrane Potential**

In preconditioned cells, we found a modest depolarization of the inner mitochondrial membrane just before index ischemia. Preconditioning with simulated ischemia decreased mitochondrial membrane potential, as reflected by the aggregate (red) signal of JC-1, to $90 \pm 3\%$ in Girardi cells (Figure 2A). The corresponding value for adenosine was $82 \pm 7\%$ and $87 \pm 4\%$ for diazoxide ($P<0.05$ versus control 100%, Figure 2A). The coadministration of 5-HD abolished the inner mitochondrial membrane depolarization that had been induced by preconditioning — simulated ischemia + 5-HD $103 \pm 5\%$, adenosine + 5-HD $90 \pm 3\%$, and diazoxide + 5-HD $94 \pm 5\%$ ($P=NS$ versus controls, Figure 2).

A second potentiometric dye, DiOC6, showed similar results (simulated ischemia $93 \pm 5\%$, adenosine $87 \pm 7\%$, diazoxide $88 \pm 1\%$, $P<0.05$, Figure 2B). Again, this depolarized phenotype was abolished by the coadministration of 5-HD (Figure 2B). The mitochondrial data using C2C12 cells are summarized in the Table for this and all subsequent measurements.

**Preconditioning Decreases Cellular ATP Levels**

Similar to the results published by Murry et al in 1986,13 preconditioned cells exhibit lower levels of ATP than controls when investigated just before index ischemia (control $0.21 \pm 0.03$ nmol/L/μg protein, simulated preconditioning ischemia $0.12 \pm 0.02$, adenosine $0.15 \pm 0.02$, diazoxide $0.11 \pm 0.02$, $P<0.05$, n=6, Figure 3). Interestingly, 5-HD, the known antagonist of preconditioning-induced cytoprotection, did not modulate cellular ATP levels when coadministered with the preconditioning triggers (simulated ischemia + 5-HD $0.13 \pm 0.02$ nmol/L/μg protein, adenosine + 5-HD $0.14 \pm 0.02$, diazoxide + 5-HD $0.14 \pm 0.01$, $P<0.05$ versus control cells, Figure 3).

**Oxygen Consumption Is Increased in Preconditioned Cells**

Control (nonpreconditioned) cells consumed oxygen at a rate of $2.3 \pm 0.1$ nmol/L oxygen/min/1×10⁶ cells. Preconditioning increased oxygen consumption (simulated ischemia $3.12 \pm 0.1$, adenosine $3.11 \pm 0.3$, diazoxide $2.6 \pm 0.2$, $P<0.05$, Figure 4). The increase in cellular oxygen consumption seen with preconditioning was abolished by the coadministration of 5-HD (simulated ischemia + 5-HD $2.05 \pm 0.1$ nmol/L oxygen/min/1×10⁶ cells, adenosine + 5-HD $2.3 \pm 0.4$, diazoxide + 5-HD $2.1 \pm 0.3$, $P=NS$ versus controls, Figure 4).

**Discussion**

The present study demonstrates that mitochondria within intact preconditioned cells enter index ischemia exhibiting features consistent with respiratory uncoupling, reflected by a decrease in inner mitochondrial membrane potential, reduced cellular ATP levels, and an increase in oxygen consumption. Thus, cells exhibiting what appears to be a less-efficient energetic state immediately before prolonged ischemia are more resistant of this lethal insult. Interestingly, the preconditioning antagonist 5-hydroxydecanoate attenuated cell viability, the modest depolarization, and the rate of oxygen consumption, but did not alter cellular ATP levels. The
cellular consequences of modulating mitochondrial function in the setting of preconditioning-induced ischemic tolerance need to be explored.

The mitochondrion is known to be a pivotal rheostat in determining cell survival versus cell death. A multitude of mitochondrial processes, including energy production, reactive oxygen species generation/signaling, and calcium homeostasis, are thought to be major contributors in this determination of cellular fate during cellular stress. How uncoupling of oxidation from phosphorylation by preconditioning can prime these mitochondria to display improved tolerance to ischemia is the major question generated by the observations demonstrated in this study.

The initial description of ischemic preconditioning implied a role for attenuated ATP depletion during ischemia as a major factor in delaying ischemic cell death. More recently, Fryer at al demonstrated that preconditioning maintained mitochondrial ATP biosynthesis following the index ischemia. Collectively, these and other data suggest that mitochondrial energetics may be central to the preconditioning program. How then could respiratory uncoupling support this hypothesis? One potential mechanism may be via uncoupling-induced rapid glucose uptake, as evident in skeletal muscle cells. The concept of enhanced glucose uptake

### Table: Preconditioning-Induced Changes in Mitochondrial Parameters in C2C12 Myotubes

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<tr>
<td>IPC + 5-HD</td>
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<td>6</td>
<td>0.24</td>
<td>0.01</td>
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*Figure 3. Preconditioning-induced changes in cellular ATP levels in Girardi cells. Using recombinant firefly luciferase bioluminescence, cellular ATP levels are shown to be decreased in preconditioning with either simulated ischemia, adenosine, or diazoxide. 5-HD had no significant effect on preconditioning-induced attenuation of ATP levels. n≥10, *P<0.05 vs non-preconditioned cells.*

*Figure 4. Oxygen consumption in preconditioned Girardi cells. Oxygen consumption measurement using a Clark-type electrode revealed that preconditioned cells exhibit increased oxygen consumption by approximately 25% to 30% compared with control cells. Coadministration of the mitochondrial KATP-channel blocker 5-HD abolished the change in oxygen consumption. n≥5, *P<0.05 vs control cells.*
and glycolytic flux following preconditioning has been demonstrated in the isolated rabbit and rat heart.\textsuperscript{17,18} Thus, hypothetically uncoupling mediated-glucose uptake may be a mechanism whereby this mitochondrial phenotype promotes ischemic tolerance.

The generation of reactive oxygen species (ROS) by the mitochondria to function as signaling intermediates in the preconditioning program has recently been described.\textsuperscript{19–21} These modulatory effects are probably mediated by a modest induction of ROS.\textsuperscript{22} Conversely, a more robust generation of these compounds can have detrimental effects on cellular survival and integrity.\textsuperscript{23,24} Thus, in the context of our findings, it would be appropriate to examine the role of respiratory uncoupling on the regulation of mitochondrial ROS generation. The role of respiratory uncoupling in the generation of ROS has recently been explored in the context of genetic manipulation of the uncoupling peptides. Upregulation or overexpression of these peptides has induced an uncoupled phenotype with a concurrent attenuation of the generation of ROS.\textsuperscript{25,26} Conversely, the genetic ablation of uncoupling peptides\textsuperscript{27,28} uniformly demonstrates increased respiratory coupling and an accompanying augmentation of ROS production. Collectively these data would be consistent with a potential role for enhanced mitochondrial respiratory uncoupling as a regulatory process that may limit excessive ROS generation in the context of ischemia.

A reduction in mitochondrial calcium overload has been demonstrated with preconditioning and is postulated to contribute to ischemic tolerance.\textsuperscript{29–31} The role of respiratory uncoupling on mitochondrial calcium homeostasis has not been extensively explored. However, NO-induced protection against cardiomyocyte ischemia and reoxygenation invokes both a modest and sustained mitochondrial depolarization and a reduction in mitochondrial Ca\textsuperscript{2+} uptake.\textsuperscript{32} Whether this mitochondrial depolarization is associated with or independent of respiratory uncoupling is not yet known.

The role of mitochondrial K\textsubscript{ATP}-channel activation on the modulation of mitochondrial function in preconditioning is being actively studied and has not yet been fully elucidated.\textsuperscript{3,5,33–36} We demonstrate in two cell lines that mitochondrial K\textsubscript{ATP} activation with dazoxe parallels both ischemic and adenosine preconditioning by inducing mitochondrial respiratory uncoupling of mitochondria in situ. Interestingly, the putative specific mitochondrial K\textsubscript{ATP}-channel antagonist 5-HD did not reverse all features of uncoupling but predominantly blunted the membrane depolarization and oxygen consumption. These data preferentially support a role for mitochondrial K\textsubscript{ATP} activation in the modulation of mitochondrial calcium homeostasis\textsuperscript{31,32,37} and in the generation of ROS.\textsuperscript{38}

Several limitations of the present study have to be acknowledged. Although 5-HD reversed the increase in oxygen consumption in our cell system, it did not have a significant impact on cellular ATP levels. Currently, we cannot offer an explanation for this finding beyond the points made in this discussion. Secondly, mitochondrial ATP synthesis, rather than whole-cell ATP levels, constitutes a possibly better indicator of mitochondrial function. In our intact cellular model, no meaningful ATP-synthesis data could be obtained.

In summary, this study demonstrates that the cell survival program activated by preconditioning induces modest, yet potentially important, mitochondrial uncoupling of in situ mitochondria. Investigating the consequences of uncoupling seems warranted to better understand the role of mitochondria in cell survival and preconditioning.

Acknowledgments

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