Mitochondria are recognized as central regulators of life and death in cardiac myocytes and a variety of other cells. A major player in the cell death pathway is the mitochondrial permeability transition pore (PTP), a nonspecific pore that allows passage of solutes up to 1.5 kDa, that opens at the contact site between outer and inner mitochondrial membranes under conditions of elevated matrix calcium. Opening of the PTP results in the loss of mitochondrial membrane potential ($\Delta \Psi_m$), massive swelling of mitochondria, rupture of the outer membrane, and release of intermembrane components that induce apoptosis. Drugs that “block” PTP, such as cyclosporin A (CsA) and bongkrekic acid (BA), inhibit both necrotic and apoptotic cell death.

We and other investigators have recently found that drugs which open mitochondrial ATP-sensitive potassium (mitoK$_{\text{ATP}}$) channels protect heart cells against ischemia/reperfusion injury and oxidative stress. By virtue of its pharmacology and its phenotypic consequences, this mode of action is distinguishable from that of other cardioprotective interventions. (Circ Res. 2003;92:195-202.)

**Key Words:** mitochondria □ potassium channels □ membrane potential □ cell death □ oxidative stress

Differential Actions of Cardioprotective Agents on the Mitochondrial Death Pathway

Masaharu Akao, Brian O’Rourke, Hideo Kusuoka, Yasushi Teshima, Steven P. Jones, Eduardo Marbán

Abstract—We examined the effect of cardioprotective agents on three distinct phases of the H$_2$O$_2$-induced response that leads to loss of mitochondrial membrane potential ($\Delta \Psi_m$) and cell death in cultured cardiac myocytes: (1) priming, consisting of calcium-dependent morphological changes in mitochondria (swelling and loss of cristae), with preserved $\Delta \Psi_m$; (2) depolarization, the rapid $\Delta \Psi_m$ depolarization caused by mitochondrial permeability transition pore (PTP) opening, and (3) cell fragmentation. The mitochondrial ATP-sensitive potassium (mitoK$_{\text{ATP}}$) channel opener diazoxide markedly decreased the likelihood that cells would undergo priming: many mitochondria remained fully polarized and morphologically intact. Diazoxide not only decreased the number of cells undergoing $\Delta \Psi_m$ depolarization but also delayed the onset of $\Delta \Psi_m$ loss, whereas it did not change the duration of depolarization in unprotected cells. The adenine nucleotide translocase inhibitor bongkrekic acid mimicked the effect of diazoxide to suppress priming, except that its effects were not blocked by the mitoK$_{\text{ATP}}$ channel blocker 5-hydroxydecanoate. In contrast, the PTP inhibitor cyclosporin A (CsA) did not prevent priming: neither latency for $\Delta \Psi_m$ depolarization nor mitochondrial morphological changes were affected. However, CsA slowed the process of depolarization and blunted its severity. Importantly, coapplication of diazoxide and CsA exhibited additive effects, improving the efficacy of protection. Activation of mitoK$_{\text{ATP}}$ channels suppresses the cell death process at its earliest stage, by preserving mitochondrial integrity during oxidative stress. From this manuscript we conclude that drugs that differentially affect mitoK$_{\text{ATP}}$ channels merit further study.
Materials and Methods

All procedures were performed in accordance with The Johns Hopkins University animal care guidelines, which conform to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

Chemicals and Reagents
All of the chemicals and reagents were purchased from Sigma, unless otherwise stated. BA was obtained from Calbiochem.

Primary Culture of Neonatal Rat Cardiac Ventricular Myocytes
Cardiac ventricular myocytes were prepared from 1- to 2-day-old Sprague-Dawley rats (Zivic Laboratories Inc, Pittsburgh, Pa) and cultured as previously described.

Assessment of $\Delta \psi_m$
$\Delta \psi_m$ level was assessed by either time-lapse confocal microscopy or fluorescence-activated cell sorter (FACS) analysis of cells stained with a fluorescent $\Delta \psi_m$ indicator, tetramethylrhodamine ethyl ester (TMRE; Molecular Probes), as previously described. In the FACS analysis, 30% of the control cells had low TMRE fluorescence, but this results from the appearance of some broken or fragmented cells due to trypsinization, and not from the poor loading of the dye.

Transmission Electron Microscopy
Cells were fixed in 2% glutaraldehyde and subjected to post fixation with 1% osmium tetroxide in 0.1 mol/L cacodylate. Formvar-coated copper grids were stained with 2% uranyl acetate, which was followed by subsequent dehydration with series of ethanol. The samples were embedded overnight with a 1:1 ratio of propylene oxide to epoxy resin. Thereafter, this solution was replaced with 100% epoxy resin and then polymerized in a dry oven at 60°C. Ultrathin sections (70-nm thick) from the embedded samples were imaged by use of a Philips CM 120 transmission electron microscope.

Image Analysis
Quantitative image analysis was performed using image analysis software (ImageJ).

Assessment of Cell Viability
For quantification of cellular viability, cells were loaded with 1 µg/mL propidium iodide (PI; Molecular Probes) at the end of experimental protocols, and PI fluorescence was analyzed at 582 nm. The number of cells that were “PI-positive” was expressed as a percentage of total cell count, and these cells were considered necrotic.

Immunoblot Analysis
Cells plated on dishes were directly lysed with Laemmli buffer (BioRad), and dissolved proteins were subjected to immunoblot analysis. Immunoblot was performed as previously described. All primary antibodies used for immunoblot were purchased from Cell Signaling Technology.

Statistical Analysis
All quantitative data are presented as mean±SEM. Multiple comparisons among groups were carried out by one-way ANOVA with Fisher’s least significant difference as the post hoc test. A level of $P<$0.05 was accepted as statistically significant.

Results
Quantitative Analysis of TMRE Histograms
Figure 1 shows representative histograms of TMRE fluorescence. We previously defined three overlapping log-normal distributions in TMRE histograms (populations I, II, and III). Population I consisted of cells with intact $\Delta \psi_m$, II of cells with dissipated $\Delta \psi_m$, and III of cells or cell fragments with very low fluorescence. The three distributions change tremendously according to the experimental conditions. At baseline, population I predominates; oxidant stress shifts the cells largely into II and III. Diazoxide protected against $H_2O_2$-induced loss of $\Delta \psi_m$, as evidenced by the preservation of the cells belonging to population I. The effects of various other pharmacological agents further define the distributions and their properties, as discussed below.

To render these observations quantitative, we assumed that the TMRE fluorescence in each population of cells (I, II, and III) follows a log-normal distribution, in which case the population histogram would represent the summation of three overlapping distributions. The parameters of those distributions were determined from best fits to the actual data. Log scales of the x-axis in TMRE fluorescence were converted to a linear scale (10$^1$: 1, 10$^3$: 1024), and the parameters of each fitted normal distribution (mean and SD), and the fractions belonging to each population were obtained. Examples illustrating the curve fitting approach are shown in Figure 1, and pooled results are shown in the Table.
Differential Actions of Cardioprotective Agents: mitoK<sub>ATP</sub> Channel Opener and PTP Blockers

The concept of three distinct phases (priming, depolarization, and fragmentation) in the response to oxidants would be much more useful if the various phases were to differ in their responsiveness to defined pharmacological interventions. To determine whether this was the case, we used blockers and activators of mitoK<sub>ATP</sub> channels and PTP. Figure 2A shows FACS data as representative histograms of TMRE fluorescence from various experimental groups. Diazoxide protected against H<sub>2</sub>O<sub>2</sub>-induced loss of ΔΨ<sub>mem</sub>, and the protective effect of diazoxide was abolished by the mitoK<sub>ATP</sub> channel blocker.

### Table: Statistical Parameters of Each Population

<table>
<thead>
<tr>
<th></th>
<th>P-I</th>
<th>P-II</th>
<th>P-III</th>
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<tbody>
<tr>
<td>Mean</td>
<td>652</td>
<td>479</td>
<td>297</td>
</tr>
<tr>
<td>SD</td>
<td>50.0</td>
<td>77.2</td>
<td>64.9</td>
</tr>
<tr>
<td>Percent</td>
<td>62.3</td>
<td>57.7</td>
<td>7.6</td>
</tr>
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- FL-2 histogram curves were fitted with 3 overlapping populations (P-I, P-II, and P-III), which follow the log-normal distribution, and the parameters of each fitted normal distribution (mean and SD) and the fractions of cells belonging to those populations were obtained.

**Figure 2.** Effects of cardioprotective agents on ΔΨ<sub>mem</sub> and SSC. A, FL-2 histograms of FACS data from TMRE-loaded cells are shown. Cells were pretreated with various drugs for 30 minutes, followed by treatment with 100 μmol/L H<sub>2</sub>O<sub>2</sub> for 2 hours at 37°C. DZ indicates diazoxide 100 μmol/L; CsA, 0.2 or 1.0 μmol/L; BA, 50 μmol/L; SHD, 500 μmol/L; and Pina, pinacidil 100 μmol/L. In all of the histograms, the positions of the peak in populations I and II were indicated by vertical dashed lines. Results are representative data from at least 3 independent experiments. B, Summarized data of the percentage of cells that maintain high (>10<sup>2</sup>) TMRE fluorescence from at least 3 independent experiments (n=5 or 6 for all groups). *P<0.05 vs H group; #P<0.05 vs corresponding SHD-free group. C, Density plots of FACS data from TMRE-loaded cells: FL-2 versus SSC. The distributions of cells were divided into 3 populations: I, II, and III (gray ellipses). D, Summarized data of the percentage of cells with PI-positive (n=4 for all groups). *P<0.05 vs H group.
5-hydroxydecanoate (5-HD, 500 µmol/L). These observations with TMRE confirm our previous findings with a different \( \Delta \Psi_m \) probe, JC-1. \(^{14}\) Diazoxide (DZ) preserved a sizable population of cells with a normal \( \Delta \Psi_m \) (note the prominent population I in the histogram labeled DZ); addition of 5-HD prevented the salutary effect of diazoxide, shifting cells into populations II or III (DZ+5HD).

One of our major interests was in comparing the effects of mitoK\(_{\text{ATP}}\) channel activators such as diazoxide with those of PTP blockers. Unlike diazoxide, the PTP blocker CsA (0.2 or 1.0 µmol/L) did not prevent \( \Delta \Psi_m \) loss but shifted the mean fluorescence of population II rightward toward a state of partial depolarization (CsA0.2, CsA1.0). CsA also decreased the fraction of cells in population III. On the other hand, BA (50 µmol/L), an inhibitor of adenine nucleotide translocase (a putative component of the PTP), \(^{18}\) prevented the \( \text{H}_2\text{O}_2 \)-induced loss of \( \Delta \Psi_m \) in a manner similar to that of diazoxide, exhibiting preservation of population I. The effects of neither PTP blocker could be antagonized by 5-HD. Notably, coapplication of diazoxide and CsA (1.0 µmol/L) had an additive effect, as evidenced by the rightward shift of population II fluorescence and the enhanced preservation of population I. In addition, another K\(_{\text{ATP}}\) channel opener, pinacidil (100 µmol/L), also protected myocytes by preserving population I. Figure 2B shows pooled data for the percentage of cells that retain a high TMRE fluorescence (defined as >10\(^{\text{th}}\) in this analysis) in various experimental groups. These data indicate that diazoxide is effective in limiting the extent of \( \Delta \Psi_m \) depolarization due to \( \text{H}_2\text{O}_2 \) and unique in that the protection is 5-HD–sensitive. The effect of CsA was also concentration-dependent but not blocked by 5-HD. Coapplication of diazoxide and CsA exhibited an additive overall effect.

As another means of assessing the effects of diazoxide and CsA, we fitted the TMRE histograms to normal distributions and determined the best-fit parameters (Table). The protective effect of diazoxide was primarily evidenced by the increased number of cells in population I. The phenotype of the CsA-treated groups was distinctly different: population II was shifted rightward, as shown by the increase in the mean value of II. The difference in percentage of cells in each population between the diazoxide and CsA groups was statistically significant (\( P<0.001 \), by \( \chi^2 \) test). The protective effect of CsA was evident here, in a concentration-dependent manner, from the shift of population II to higher fluorescence values as well as the modest increase in amplitude of population I. Coapplication of diazoxide and CsA exerted both beneficial effects.

The differential mode of action between diazoxide and CsA is further evident from the FL-2/side-scatter (SSC) density plots of Figure 2C. SSC is a parameter of cellular morphology in FACS analysis that represents mitochondrial swelling, as discussed below. Pretreatment with diazoxide decreased the number of cells that underwent \( \Delta \Psi_m \) loss, keeping the population of cells in a fully polarized state with appreciable preservation of SSC. In contrast, CsA did not block \( \Delta \Psi_m \) loss, but blunted its severity, as evidenced by the presence of a rightward-shifted population II and a remarkably smaller population III. As before, the additive effect of diazoxide and CsA is clearly demonstrated by the presence of the shift in population II as well as the preservation of population I.

To assess the viability of cells, PI exclusion was analyzed by FACS. Both diazoxide and CsA resulted in significant protection against this index of necrosis induced by \( \text{H}_2\text{O}_2 \) (Figure 2D), supporting the concept that PTP opening is responsible for necrosis as well as apoptosis, \(^{10}\) at least in this in vitro model.

To characterize the pharmacological effects on a single-cell basis, time-lapse confocal microscopy was performed with TMRE-loaded myocytes. Figure 3A shows the time-dependent changes of mean TMRE fluorescence from each individual cell. Diazoxide not only decreased the number of cells undergoing dissipation of \( \Delta \Psi_m \) but also delayed the onset of \( \Delta \Psi_m \) loss, whereas it did not change the duration of \( \Delta \Psi_m \) loss in unprotected cells. In contrast, CsA modestly decreased the number of cells that lost \( \Delta \Psi_m \) and did not delay the onset of \( \Delta \Psi_m \) loss, but prolonged the duration of \( \Delta \Psi_m \) loss. BA appeared to be protective in a manner similar to diazoxide. Mean TMRE fluorescence levels from the 50 cells were measured at 60 minutes, and the data from 4 to 6 independent experiments are summarized in Figure 3B. Consistent with the FACS data in Figures 2A and 2B, the mitoK\(_{\text{ATP}}\) channel blocker 5-HD did not abolish the effects of CsA or BA. Analysis of the cumulative first latency to \( \Delta \Psi_m \) loss for individual cells reveals that only diazoxide delayed the onset of \( \Delta \Psi_m \) loss (Figure 3C). Figure 3D indicates the duration required for \( \Delta \Psi_m \) loss in each group. In contrast with the rapid \( \Delta \Psi_m \) loss (\(<5\) minutes) in both diazoxide and BA groups, CsA prolonged the dissipating process of \( \Delta \Psi_m \).

The differential effects of diazoxide and CsA on SSC were further investigated. We previously showed that the decrease in SSC reflects progressive mitochondrial swelling. \(^{16}\) Figure 4 shows the results from transmission electron microscopy. Diazoxide remarkably prevented the \( \text{H}_2\text{O}_2 \)-induced changes in mitochondrial morphology, as demonstrated by the marked decrease in swelling and loss of cristae, whereas CsA did not. Quantitative analysis in Figure 4B confirmed these observations. \( \text{H}_2\text{O}_2 \) exposure caused extensive swelling of mitochondria, as reported by the increases in area and circularity (where circularity is defined as \( 4\pi \cdot [\text{area}/(\text{perimeter})^2] \)), which quantifies the roundness of mitochondria), and loss of cristae. Diazoxide significantly suppressed all of those detrimental morphological alterations, thereby preserving mitochondrial integrity. In contrast, CsA did not protect mitochondrial morphology.

**Roles of Akt and MAPK Pathways in mitoK\(_{\text{ATP}}\) Channel-Mediated Protection**

Akt, a serine-threonine kinase and also known as protein kinase B, is a powerful survival signal. \(^{19}\) Adenoviral gene transfer of constitutively active Akt inhibits cardiac myocyte apoptosis during ischemia/reperfusion in vitro and in vivo.\(^{20,21}\) In addition, other signaling cascades such as the mitogen-activated protein kinase (MAPK) superfamily (c-Jun N-terminal kinases [JNKs], p38-MAPK, and extracellularly responsive kinases [ERKs]), which are activated in response to reactive oxygen species (ROS), have been implicated in a number of adaptive and maladaptive processes, including...
hypertrophy, alteration of gene expression, cell survival, and cell death in the cardiovascular system. The involvement of JNK in the regulation of cell death has attracted especially keen interest. Therefore, we investigated whether these signaling cascades are somehow related to mitoKATP channel-mediated protection. Figure 5 shows the data for immunoblot analysis against the phosphorylated form of Akt (P-Akt) and three MAPKs (P-JNK, P-p38 MAPK, and P-ERK). Exposure to H2O2 induced acute and transient phosphorylation of Akt and all MAPKs, which returned to normal level within 60 minutes. Diazoxide blunted the phosphorylation of Akt in a 5-HD–inhibitable manner, but not that of MAPKs. Quantitative measurements of band density are shown in Figure 5B. The observed changes in Akt are in the wrong direction to...
explain cytoprotection by diazoxide. Thus, the effects of mitoK<sub>ATP</sub> channels are not mediated by Akt or MAPK activation.

**Discussion**

**Differential Actions of Cardioprotective Agents on the Mitochondrial Death Pathway**

Cardiac cells undergoing ΔΨ<sub>m</sub> loss and eventual cell death under oxidant stress exhibit three distinct phases: priming, depolarization, and fragmentation. The protective effect of diazoxide is due to an inhibition of priming; the observed reduction of depolarization and fragmentation are proposed to be beneficial downstream consequences (Figure 6). Diazoxide preserved a sizable population of cells with a normal ΔΨ<sub>m</sub> (population I); however, it did not affect the transition between populations II and III (Figures 1 and 2; Table). Time-lapse confocal analyses showed that diazoxide not only decreased the number of cells undergoing dissipation of ΔΨ<sub>m</sub> but also delayed the onset of ΔΨ<sub>m</sub> loss in unprotected cells (Figures 3A and 3C), further supporting the idea that diazoxide blocks priming. However, diazoxide did not change the duration of ΔΨ<sub>m</sub> loss in unprotected cells (Figures 3A and 3D). This protective effect of diazoxide on ΔΨ<sub>m</sub> is concentration-dependent and can be inhibited by the mitoK<sub>ATP</sub> channel blocker 5-HD. The effect of opening mitoK<sub>ATP</sub> channels on priming was further confirmed by testing the effects of other mitoK<sub>ATP</sub> channel openers, nicorandil and pinacidil, in the same system. Nicorandil protected myocytes in a similar manner to diazoxide; it preserved population I and delayed or inhibited the onset of ΔΨ<sub>m</sub> loss triggered by...
Our previous findings that diazoxide attenuates matrix Ca²⁺ influx into mitochondria and that diazoxide preserves mitochondrial function was also demonstrated by 5-HD. Moreover, activation of mitoK ATP channels block priming is consistent with our previous findings that diazoxide attenuates matrix Ca²⁺ overload during simulated ischemia. Given that the priming process features Ca²⁺-dependent changes in mitochondrial morphology, we examined whether diazoxide has protective effects on ultrastructure. As shown in Figure 4, diazoxide strikingly inhibited the swelling and loss of cristae caused by H₂O₂. The possible involvement of mitoK ATP channels in the preservation of mitochondrial function was also demonstrated elsewhere.

Studies from our laboratory and that of Garlid and colleagues supported the original concept that diazoxide and 5-HD selectively act on mitoK ATP channels in heart cells. Diazoxide is known to inhibit succinate dehydrogenase and to decrease the rate of succinate-supported respiration in heart mitochondria. Moreover, 5-HD, a hydroxyl derivative of medium-chain fatty acids, may be metabolized to generate 5-HD coenzyme A, perhaps bypassing the block of respiration.

This has generated the alternative hypothesis that respiratory inhibition might underlie the cardioprotection afforded by diazoxide. Although the focus of the present study is not to determine the molecular target of diazoxide and 5-HD, our observations suggest that the effects of these agents cannot be explained by the modulation of mitochondrial respiration. Direct inhibition of succinate dehydrogenase by 3-nitropropionic acid or malonate (100 to 1,000 µmol/L) did not have any appreciable protective effects on mitochondrial function, assessed using TMRE fluorescence (S.P. Jones, M. Akao, and E. Marbán, unpublished data, 2002).

Nicorandil, which was similarly cardioprotective, has no known inhibitory effects on respiration. We also showed that glibenclamide, a classical K ATP channel blocker, abolished the protective effects of diazoxide, a fact that cannot be explained by the bypassing of the block of respiration. Indeed, glibenclamide has been reported to inhibit mitochondrial respiration. Taken together, these data argue that the only common feature of these compounds associated with protection is their ability to increase mitochondrial K⁺ influx (or block it in the case of inhibitors). Therefore, activation of a completely different class of mitochondrial K⁺ channel has recently been shown to be cardioprotective.

In contrast to diazoxide, CsA did not prevent priming, but slowed the process of depolarization and blunted its severity (Figure 6). CsA had a minor effect in preserving population I; instead, it shifted population II toward a partially depolarized state and prevented the transition between populations II and III (Figure 2, Table). Time-lapse analyses showed that CsA did not affect the latency of depolarization (Figures 3A and 3C) but slowed the rate of ΔΨ𝑚 depolarization and left the cells partially depolarized (Figures 3A and 3D). The effect of CsA was also concentration-dependent (Figure 2B, Table) but was not blocked by 5-HD (Figures 2A, 2B, and 3B). Consistent with the idea that CsA does not affect priming, CsA did not prevent the morphological changes in mitochondria (Figure 4). Nevertheless, the partial depolarization that persists in the presence of CsA does not suffice to trigger cell death. These differential effects of diazoxide and CsA rationalize their additive effects.

The block of PTP by CsA reveals the existence of CsA-insensitive pathways that produce slow and partial mitochondrial depolarization in response to oxidative stress (Figure 6, dashed arrow). Indeed, several studies have implicated other low-conductance CsA-insensitive channels in the inner mitochondrial membrane in ΔΨ𝑚 depolarization. Alternatively, a low-conductance state of the PTP may be involved. Whatever the mechanism of the partial depolarization, it clearly differs from the conventional depolarization under drug-free conditions in various key respects. First, depolarization in CsA is slow and incomplete. Second, the concomitant increase of population III that accompanies full depolarization is not observed; indeed, it is statistically suppressed (Table). Finally, cells appear to be able to recover from the incomplete depolarization in CsA; this conjecture is based not on our own observations but rather on the well-documented efficacy of CsA in blunting apoptosis.

Unexpectedly, another PTP blocker (BA) resembled diazoxide in that it inhibited the priming phase, albeit not in a 5-HD–inhibitable manner (Figures 2 and 3). The difference in the effects of CsA and BA presumably reflects the different molecular targets of those agents. CsA is a ligand of cyclophilin D and BA binds to adenine nucleotide translocase, both of which are putative molecular components of the PTP complex. BA, however, is also likely to have major effects on oxidative phosphorylation, because it will inhibit mitochondrial ADP uptake via adenine nucleotide translocase, thereby inhibiting ADP-stimulated respiration. This consequently could alter mitochondrial ROS production and eliminate amplification of the H₂O₂ effect by mitochondrial ROS-induced ROS release.

Involvement of Akt and MAPK Pathways in mitoK ATP Channel–Mediated Protection

Akt, a potent mediator of cell survival pathway, was not further activated under diazoxide treatment; instead, the phosphorylation of Akt was decreased. Thus, Akt phosphorylation is not causally involved in diazoxide-mediated protection. The downregulation of Akt may be the result of attenuation of the insulting stimulus due to the protection...
afforded by diazoxide. This finding also suggests that the protective effect of diazoxide starts to operate quite early, because the phosphorylation of Akt was blunted as soon as 2 minutes after H$_2$O$_2$ exposure. This acute action of diazoxide is consistent with our proposed model, in which diazoxide acts exclusively on the priming phase. Meanwhile, diazoxide did not affect the acute phosphorylation of MAPKs. Therefore, the protective effect of diazoxide against H$_2$O$_2$-induced cell death is not due to a mere radical-scavenging effect, but rather to a selective modulation of intracellular signaling pathways that come into play immediately after insulting stimuli.

Clinical Implications
In the present study, we have provided evidence for selective and also additive protection of mitochondria by diazoxide and CsA in the mitochondrial death pathway. These novel findings are of potential importance from the standpoint of improving the efficacy of therapeutic strategies against diseases in which oxidative stress is involved.

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