Mitochondrial K$_{ATP}$ channel activation reduces anoxic injury by restoring mitochondrial membrane potential

MEIFENG XU, YIGANG WANG, AHMAR AYUB, AND MUHAMMAD ASHRAF
Department of Pathology and Laboratory Medicine, University of Cincinnati, Cincinnati, Ohio 45267-0529

Received 27 April 2001; accepted in final form 17 May 2001

Mitochondrial K$_{ATP}$ channel activation reduces anoxic injury by restoring mitochondrial membrane potential. Am J Physiol Heart Circ Physiol 281: H1295–H1303, 2001.—Mitochondrial membrane potential ($\Delta$V$_m$) is severely compromised in the myocardium after ischemia-reperfusion and triggers apoptotic events leading to cell demise. This study tests the hypothesis that mitochondrial ATP-sensitive K$^+$ (mitoK$_{ATP}$) channel activation prevents the collapse of $\Delta$V$_m$ in myocytes during anoxia-reoxygenation (A-R) and is responsible for cell protection via inhibition of apoptosis. After 3-h anoxia and 2-h reoxygenation, the cultured myocytes underwent extensive damage, as evidenced by decreased cell viability, compromised membrane permeability, increased apoptosis, and decreased ATP concentration. Mitochondria in A-R myocytes were swollen and fuzzy as shown after staining with Mito Tracker Orange CMTMRos and in an electron microscope and exhibited a collapsed $\Delta$V$_m$, as monitored by 5,5'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carboxyanine iodide (JC-1). Cytochrome c was released from mitochondria into the cytosol as demonstrated by cytochrome c immunostaining. Activation of mitoK$_{ATP}$ channel with diazoxide (100 $\mu$mol/l) resulted in a significant protection against mitochondrial damage, ATP depletion, cytochrome c loss, and stabilized $\Delta$V$_m$. This protection was blocked by 5-hydroxydecanoate (500 $\mu$mol/l), a mitoK$_{ATP}$ channel-selective inhibitor, but not by HMR-1098 (30 $\mu$mol/l), a putative sarcolemmal K$_{ATP}$ channel-selective inhibitor. Dissipation of $\Delta$V$_m$ also leads to opening of mitochondrial permeability transition pores, which was prevented by cyclosporin A. The data support the hypothesis that A-R disrupts $\Delta$V$_m$ and induces apoptosis, which are prevented by the activation of the mitoK$_{ATP}$ channel. This further emphasizes the therapeutic significance of mitoK$_{ATP}$ channel agonists in the prevention of ischemia-reperfusion cell injury.

apoptosis; myocytes; ATP; permeability transition pore; cytochrome c

MITOCHONDRIA ARE MAJOR MYOCYTE organelles, and they play an important role in cell life and death. It is well known that myocardial ischemia-reperfusion (I/R) induces significant pathological changes in mitochondria (17). The impaired mitochondrial function after I/R is due to imbalance of cytosolic ions, electron transport, production of free radicals, and alteration of membrane potential (15, 28, 32), eventually leading to apoptosis in the ischemic myocardium (2, 5, 29).

Mitochondrial membrane potential ($\Delta$V$_m$) originates from the asymmetric distribution of protons across the inner mitochondrial membrane and is essential for the maintenance of mitochondrial function. The relationship between $\Delta$V$_m$ and pathological conditions such as anoxia and apoptosis was the topic of several recent studies (8, 18, 20, 37). $\Delta$V$_m$ is compromised due to the opening of permeability transition pores at an early stage of apoptosis, whereas $\Delta$V$_m$ is needed for mitochondrial ATP production during apoptosis (36).

It has been reported (11, 22, 34, 35) that ATP-sensitive K$^+$ (K$_{ATP}$) channel openers exert cardioprotective effects in various animal models of ischemia-reperfusion. According to these studies, the mitochondrial K$_{ATP}$(mitoK$_{ATP}$) channel-selective agonist diazoxide improved postischemic functional recovery in isolated rabbit and rat hearts (11, 34). It has been indicated that diazoxide attenuated I/R injury due to preservation of mitochondrial function (16). 5-Hydroxynonadecanoate (5-HD), a mitoK$_{ATP}$ channel blocker, blocked cardioprotection by diazoxide (11, 35). Moreover, mitoK$_{ATP}$ openers depolarized mitochondrial membrane potential by 10 mV (13). It is likely that the mechanism of cardioprotection against ischemic injury by mitoK$_{ATP}$ channel may involve stabilization of $\Delta$V$_m$. To address this question, the effect of mitoK$_{ATP}$ channel on $\Delta$V$_m$ as well as cytochrome c release and apoptosis was investigated.

MATERIALS AND METHODS

Diazoxide, mouse monoclonal anti-cytochrome c, and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Fab fragment) were purchased from Sigma (St. Louis, MO). 5-HD was purchased from ICN Biomedical (Costa Mesa, CA). Cyclosporin A was purchased from Biomedical Research Labs (Plymouth Meeting, PA). HMR-1098 was a gift from Dr. Garrett Gross (Milwaukee, WI). All fluorescent dyes were purchased from Molecular Probes (Eugene, OR).

Experimental Protocols

Primary myocyte-rich cultures of the neonatal rat myocytes were prepared as described previously (36). Briefly,
ventricles from hearts of 1- to 2-day-old rats were dissociated with trypsin and collagenase. The cells were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 U/ml each of penicillin and streptomycin. To selectively enrich the myocytes, dissociated cells were preplated for 2 h to allow nonmyocytes to attach to the bottom of the culture dish. The resultant suspension of myocytes was transferred onto collagen-coated 60-mm or 100-mm culture dishes. Bromodeoxyuridine (100 μM) was added during the first 24–36 h to prevent proliferation of nonmyocytes. The experiments were performed on day 3 of culture, and myocytes were divided into the following six groups (Fig. 1).

**Group 1: control.** The myocytes were incubated in Tyrode solution with glucose (25 mmol/l) during the entire experimental period.

**Group 2: A-R.** To induce complete anoxia, Tyrode solution was deoxygenated by bubbling with purified nitrogen for 1 h before the experiments. Myocytes were exposed to anaerobic glucose-free Tyrode solution and placed into the anoxic chamber (Forma 1025 anaerobic system) for 3 h of anoxia and then kept in normal Tyrode solution and returned to the CO2 incubator for 2 h of reoxygenation.

**Group 3: diazoxide + A-R.** The myocytes were preincubated with diazoxide (100 μmol/l) for 20 min before A-R.

**Group 4: 5-HD + diazoxide + A-R.** The myocytes were preincubated first with 5-HD (500 μmol/l) for 10 min and then with 5-HD and diazoxide for 20 min before A-R.

**Group 5: HMR-1098 + diazoxide + A-R.** The myocytes were preincubated first with HMR-1098 (30 μmol/l) for 10 min and then with HMR-1098 and diazoxide for 20 min before A-R.

**Group 6: cyclosporin A + A-R.** The myocytes were preincubated with cyclosporin A (5 μmol/l), an inhibitor of mitochondrial permeability transition, for 20 min before A-R. This group was included to determine whether ΔΨm has any effect on the opening of mitochondrial permeability transition pore.

**Measurement of Cell Viability, Lactate Dehydrogenase, and ATP**

Cell viability was calculated by dividing the number of trypan blue negative cells by the total number of cells examined and then multiplying by 100%. ATP was extracted by 6% trichloroacetic acid and analyzed at 340 nm in a Beckman spectrophotometer by using an ATP detection kit (Sigma). Lactate dehydrogenase (LDH) release from myocytes was measured by using a LDH detection kit (Sigma).

**Detection of Apoptosis and Distribution of Cytochrome c**

To visualize apoptotic nuclei in cardiac myocytes in situ, the ApoTag in situ apoptosis detection kit (Oncor) was used. The cultured myocytes were fixed in 4% paraformaldehyde (pH 7.4) and subjected to TdT-mediated dUTP nick-end labeling (TUNEL) assay (36).

The release and distribution of cytochrome c in intact myocytes were assayed as described by Xu et al. (36). Cultured myocytes on coverslips were fixed in 2% formaldehyde and blocked with 10% normal goat serum. Cells were stained with mouse monoclonal anti-cytochrome c as the primary antibody and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G as the secondary antibody. For mitochondrial staining, unfixed cells were incubated with 500 μmol/l Mito Tracker Orange CMTMRos for 30 min at 37°C. After being washed and then fixed in 2% formaldehyde, the cells were observed with the use of a laser scanning confocal microscope (LSM 510, Zeiss).

**Mitochondrial Morphology**

The mitochondrial ultrastructure was assessed by transmission electron microscopy. Myocytes cultured on the coverslips were immersed in 2.5% buffered glutaraldehyde and rinsed in 0.1 mol/l sodium cacodylate buffer (pH 7.3). The cells were embedded in epon resin and cut into 600-nm-thick sections with a Sorvall MTB2 ultramicrotome. The sections
were stained with uranyl acetate and lead citrate and examined with a Hitachi H-600 electron microscope at 75 kV.

Mitochondrial Membrane Potential

The changes in $\Delta \Psi_m$ were monitored with the dye 5,5',6,6''-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (24). Cells were stained with JC-1 (5 $\mu$mol/l) at 37°C for 15 min and rinsed three times with Tyrode solution. The observation was made by using a laser scanning confocal microscope. JC-1 monomer (green) fluorescence was observed by excitation with the 488-nm laser and examination of the emissions from 505 to 530 nm. JC-1 aggregate (red) fluorescence was observed by excitation with the 543-nm laser and examination of the emissions over 560 nm.

One hundred or more areas were selected from each image and the average intensity for each region was quantified (Metamorph, Universal Imaging; West Chester, PA). The ratio of JC-1 monomer to aggregate intensity for each region was calculated. An increase in this ratio was interpreted as decrease of $\Delta \Psi_m$, whereas a decrease in the ratio was interpreted as gain in $\Delta \Psi_m$ (31).

Statistical Analysis

All data, except for the $\Delta \Psi_m$ data, were obtained in at least three independent experiments with replicates of two or four for each condition. The $\Delta \Psi_m$ data were obtained from 2–3 experiments, and 10–14 images were analyzed in each group. Each image used for the $\Delta \Psi_m$ data contained over 10 myocytes and 100 areas. All data were expressed as means ± SE. Statistical significance between groups was determined by Student's $t$-test. A value of $P < 0.05$ was considered significant.

RESULTS

MitoK$_{\text{ATP}}$ Channel Activation Prevents A-R Injury

The protection by diazoxide on A-R-induced myocyte damage is shown in Figs. 2–4. Ninety percent of the control cells excluded trypan blue, whereas, in the A-R group, only 35% of the myocytes were viable (Fig. 2). LDH release (Fig. 3A) was significantly increased and ATP content (Fig. 3B) was significantly depleted after myocytes underwent A-R. Diazoxide-induced activation of the mitoK$_{\text{ATP}}$ channel before A-R reduced cell death, decreased LDH release, and preserved ATP content. The protection provided by diazoxide was similar to that of cyclosporin A.

To test whether diazoxide was activating K$_{\text{ATP}}$ channels that were in the mitochondria or in the sarcolemma, the effect of diazoxide was examined in the presence of 5-HD, an inhibitor of mitoK$_{\text{ATP}}$ channel, or HMR-1098, a sarcolemmal K$_{\text{ATP}}$ channel inhibitor. The protective effect of diazoxide was decreased in the presence of 5-HD but not in the presence of HMR-1098.

MitoK$_{\text{ATP}}$ Channel Activation Prevents Apoptosis

TUNEL assay was used to determine A-R-induced apoptosis. Less than 10% of the control myocytes had...
TUNEL-positive nuclei (Fig. 4). A-R significantly increased the number of TUNEL-positive nuclei. Diazoxide pretreatment reduced TUNEL-positive nuclei by 50%. The protective effect of diazoxide was similar to that of cyclosporin A.

It has been indicated that cytochrome c release from mitochondria is followed by apoptosis. Cytochrome c immunostaining (Fig. 5B) coincided with the distribution of mitochondria in control myocytes (Fig. 5A). After A-R, there was diffuse cytochrome c immunostaining in some cells, which suggested the release of cytochrome c into the cytosol (Fig. 5E). Pretreatment of myocytes with diazoxide significantly blocked the release of cytochrome c (Fig. 5H). 5-HD reversed the action of diazoxide and HMR-1098 did not significantly inhibit the protection by diazoxide.

**MitoK\textsubscript{ATP} Channel Activation Protects Mitochondrial Morphology**

When examined with the electron microscope, mitochondria were observed in rows between myofibrils or were scattered loosely throughout the cytoplasm (Fig. 6A). After A-R, mitochondria became swollen and cristae were disrupted and electron dense deposits (arrowhead) were observed within mitochondria (M). Glycogen was reduced. Magnification, \( \times 12,300 \). C: myocytes after DZ treatment showing well-preserved nucleus (N), mitochondria (m), and abundant glycogen (arrow). Magnification, \( \times 14,000 \). D: myocytes pretreated with cyclosporin A before A-R showing well-preserved mitochondria, nucleus (N), and glycogen (arrow). Magnification, \( \times 11,700 \).

**MitoK\textsubscript{ATP} Channel Activation Restores \( \Delta \Psi \text{m} \)**

When control myocytes were loaded with JC-1, they exhibited a heterogeneous distribution of mitochondria with low (green fluorescence of monomer) and high (red fluorescence of J-aggregates).
fluorescence of J-aggregate) $\Delta \Psi_m$. Green fluorescent mitochondria were localized near the nucleus, whereas red fluorescent mitochondria were confined to the cell periphery (Fig. 7C).

Immediately after shorter A-R (i.e., 2-h anoxia and 2-h reoxygenation), the first response of mitochondria was hyperpolarization (unpublished observations). After A-R with a longer anoxic period (3 h), myocytes...
showed marked changes in $\Delta \Psi_m$. Many myocytes displayed a loss or collapse of $\Delta \Psi_m$ as evident from the disappearance of red or both red and green fluorescence in several cells (Fig. 7F) or mitochondria were condensed into an extremely packed mass (Fig. 7E). These changes were accompanied by apoptosis-associated morphological changes, such as nuclear chromatin condensation, the reduction of cell volume (Fig. 6), and the release of cytochrome $c$ (Fig. 5). Many other myocytes displayed elongated mitochondria in the cell periphery and highly polarized mitochondria in the cell center (Fig. 7, D and F). Pretreatment of the myocytes with diazoxide protected mitochondria from the loss of $\Delta \Psi_m$ and from hyperpolarization (Fig. 7, G–I).

Whereas many cells displayed a loss or collapse of $\Delta \Psi_m$, many other cells (perhaps less damaged) displayed an increase in $\Delta \Psi_m$ in response to A-R. The ratio of JC-1 monomer (green) to aggregate (red) fluorescence was used to quantify $\Delta \Psi_m$ in these less damaged cells. Myocytes with extremely packed mitochondria and myocytes lacking red fluorescence were considered severely damaged and were excluded from analysis. With A-R, the JC-1 ratio was reduced ($\Delta \Psi_m$ increased) compared with the control myocytes (Fig. 8). The JC-1 ratio in diazoxide-pretreated cells was maintained at a level that was similar to that of control and of cyclosporin A-pretreated cells (Fig. 8). The diazoxide-induced maintenance of $\Delta \Psi_m$ was reduced by the mitoKATP inhibitor 5-HD but not by the sarcolemmal KATP channel inhibitor HMR-1098.

**DISCUSSION**

Mitochondria are the critical organelle for myocyte cell survival. A compromise of mitochondrial function during A-R may lead to cell demise. The results of this study strongly support the notion that $\Delta \Psi_m$ is severely disrupted during A-R and is accompanied by leakage of cytochrome $c$, apoptosis, destruction of cristae, and accumulation of Ca$^{2+}$ in mitochondria.

Our data indicate that reoxygenation after anoxia produced a variable but often profound loss of $\Delta \Psi_m$. A loss in $\Delta \Psi_m$ is accompanied by cytochrome $c$ release from the mitochondria and leads to induction of apoptosis in different cell types (1, 28). Loss of $\Delta \Psi_m$ is correlated with the release of cytochrome $c$ from mitochondria as determined by immunostaining. Therefore, it is highly likely that loss or dissipation of $\Delta \Psi_m$ mediated the release of cytochrome $c$, which activated apoptosis. Bialik et al. (3) also presented similar evidence of a mitochondrial apoptotic pathway during ischemia. Postanoxic reoxygenation also caused a significant elevation of intramitochondrial Ca$^{2+}$, resulting in loss of $\Delta \Psi_m$ (7, 10).

In our study, many cells indicated high polarized mitochondria after A-R. This hyperpolarization may result from lower oxygen availability or be generated by reverse ATP synthase activity supported by glycolytic ATP (4, 8). However, what seems evident is that mitochondrial high polarization is not synonymous with enhanced mitochondrial activity (9). Indeed, an inverse relationship may be assumed between the average level of mitochondrial polarization and ATP synthesis (33), at least in intact cells, where complex homeostatic mechanisms are established between mitochondria and other cytoplasmic compartments. High polarization of mitochondria may be the force to drive Ca$^{2+}$ into mitochondria, which will induce Ca$^{2+}$ overload in mitochondria and trigger apoptosis (21). Moreover, it has been reported (25) that first the mitochondrial membrane potential increases before cytochrome $c$ release that occurs after a loss in potential and mitochondrial swelling.

The present study demonstrated that diazoxide stabilized $\Delta \Psi_m$ by attenuating the loss of $\Delta \Psi_m$ and high polarization observed during A-R. Holmuhamedov et al. (13) reported that mitoKATP channel openers caused depolarization-dependent mitochondrial membrane depolarization in normal cultured myocytes. At a low concentration (100 $\mu$mol/l) of the KATP channel opener pinacidil, $\Delta \Psi_m$ of myocytes was decreased by 10 mV (13). However, a high concentration (1 mmol/l) of KATP channel opener decreased $\Delta \Psi_m$ over 150 mV. Similarly, diazoxide decreased $\Delta \Psi_m$ in mouse intact perfused pancreatic B cells and isolated liver mitochondria, accelerating the release of Ca$^{2+}$ stored in the mitochondria (12). Activation of the mitoKATP channel with diazoxide (100 $\mu$mol/l) results in K$^+$ influx, expansion of mitochondrial matrix volume, and a reduction of the inner $\Delta \Psi_m$ (13). Depolarization of the $\Delta \Psi_m$ reduced the driving force for Ca$^{2+}$ influx (6, 14), thus attenuating mitochondrial Ca$^{2+}$ overload and myocyte injury during A-R (30). Our study indicates that the stabilization of $\Delta \Psi_m$ by activation of mitoKATP channel was accompanied by remarkable recovery of ATP and absence of Ca$^{2+}$ accumulation in mitochondria.

The precise mechanism of diazoxide on $\Delta \Psi_m$ remains unknown. There are multiple electron transport systems in mitochondria that are disrupted by A-R, and opening mitoKATP channels will facilitate homeostasis of electron transport system. McPherson and Yao (23)
recently showed that δ-opioid receptor stimulation opens mitoK\textsubscript{ATP} channels, resulting in a small increase of reactive oxygen species. These reactive oxygen species are important components of mitochondrial transmembrane potential and participate in signaling cascade leading to cardioprotection. In cardiac myocytes, \( \Delta \Psi \text{m} \) may be regulated via activation of K\textsubscript{ATP} channel. Diazoxide prevented high polarization of the mitochondrial membrane and the collapse of \( \Delta \Psi \text{m} \), thus inhibiting the Ca\textsuperscript{2+} accumulation by mitochondria. This was further substantiated by use of a selective K\textsubscript{ATP} channel blocker, 5-HD, which prevented the depolarizing action of a mitoK\textsubscript{ATP} channel opener. In another study (13), it was reported that K\textsubscript{ATP} channel activation decreased ATP synthesis and released mitochondrial proteins. Both of these parameters are associated with cell death. However, overwhelming evidence (19, 20, 28) suggests that apoptosis is mediated by the cytochrome c released from the mitochondria. It is not clear how diazoxide-induced cytochrome c release can protect against ischemic injury, as reported by Holmuhamedov et al. (13). Our previous studies (36) have indicated that the concentration of cytochrome c in mitochondria displayed a negative linear correlation with the percentage of apoptosis in the myocytes. In the present study, diazoxide prevented cytochrome c release from myocytes subjected to A-R, suggesting a role of mitoK\textsubscript{ATP} channel in cardiac protection. This confirms our previous findings (30) that diazoxide inhibits both apoptosis and necrosis in late preconditioning.

The effect of diazoxide on \( \Delta \Psi \text{m} \) was comparable with that observed after cyclosporin A treatment. The reduced \( \Delta \Psi \text{m} \) increases the likelihood of opening of mitochondrial permeability transition pore which is prevented by cyclosporin A. Outward pumping of protons at mitochondrial respiratory complexes I, III, and IV generates \( \Delta \Psi \text{m} \) across the inner mitochondrial membrane (27) and decreased \( \Delta \Psi \text{m} \) facilitates opening of permeability transition pore (26). As suggested by our data, there appears to be commonality of \( \Delta \Psi \text{m} \) between opening of mitochondrial K\textsubscript{ATP} channel and cyclosporin A-sensitive permeability transition pore.

In summary, the mitoK\textsubscript{ATP} channel activation prevented the myocyte damage caused by A-R. Opening of mitoK\textsubscript{ATP} channel stabilized the \( \Delta \Psi \text{m} \) in anoxic myocytes, resulted in marked augmentation of cell ATP, and inhibited the loss of cytochrome c from mitochondria leading to attenuation of apoptosis. Thus maintenance of mitochondrial function and structural integrity by diazoxide suggests a potential therapeutic application of mitoK\textsubscript{ATP} channel agonists in preventing ischemic injury.

We are grateful to Dr. Nancy K. Kleene, Department of Cell Biology, Neurobiology, and Anatomy, University of Cincinnati, for assistance with confocal microscopy and helpful discussions.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-23597 and HL-55678.

REFERENCES


