Mitochondrial ATP-Sensitive Potassium Channel Activation Protects Cerebellar Granule Neurons From Apoptosis Induced by Oxidative Stress

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Background and Purpose—Mitochondrial ATP-sensitive potassium (mitoK_ATP) channels are present in the brain, and several reports have shown that mitoK_ATP channel openers protect the brain against ischemic injury. However, the precise mechanisms of this protection are not well established. We hypothesized that mitoK_ATP channel openers prevent apoptosis by preserving mitochondrial membrane potential.

Methods—We investigated the effect of mitoK_ATP channel openers on apoptosis induced by oxidative stress using cultured cerebellar granule neurons.

Results—The mitoK_ATP channel opener diazoxide (100 μmol/L) significantly suppressed the number of cells with terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL)–positive nuclei and the increase in caspase-3 activity induced by 20 μmol/L H_2O_2. Diazoxide and another opener, pinacidil, prevented the loss of mitochondrial inner membrane potential (ΔΨ_m) induced by H_2O_2. These effects were abolished by 5-hydroxydecanoate (500 μmol/L), a mitoK_ATP channel blocker. Cyclosporin A and bongkrekic acid, inhibitors of the mitochondrial permeability transition pore, also prevented ΔΨ_m loss, confirming the involvement of the mitochondrial permeability transition in the apoptotic cascade in neurons. Furthermore, diazoxide prevented the increase in extracellular glutamate concentration induced by H_2O_2, but this effect was not attributable to activation of surface K_ATP channels.

Conclusions—MitoK_ATP channel openers inhibited apoptosis by preserving mitochondrial inner membrane potential. These beneficial effects may suggest a possible new target for neuroprotection. (Stroke. 2003;34:1796-1802.)

Key Words: apoptosis ■ mitochondria ■ neurons ■ oxidative stress ■ potassium channels

Opening of mitochondrial ATP-sensitive potassium (mitoK_ATP) channels is protective against cardiac ischemia.1,2 The mitoK_ATP channel opener diazoxide inhibits activation of the mitochondrial apoptotic pathway induced by hydrogen peroxide (H_2O_2) in cardiac myocytes, and this protective effect is abolished by the mitoK_ATP channel blocker 5-hydroxydecanoate (5HD).3 These facts suggest that the prevention of apoptosis may be an important mechanism of mitoK_ATP-related cardioprotection. Despite these observations in the heart, less is known about the role of mitoK_ATP channels in other organs.

Apoptosis contributes to ischemic injury in the brain,4 and brain mitochondria contain 7 times more mitoK_ATP channels than heart mitochondria.5 Several reports have shown that mitoK_ATP channel openers protect the brain against ischemic injury in vivo: diazoxide preserved neuronal-vascular response after cerebral ischemia/perfusion in piglets,6 mitigated canine brain injury induced by hypothermic circulatory arrest,7 and decreased cortical infarct size in a murine stroke model.8 However, the mechanisms of these beneficial effects are still obscure. We hypothesized that mitoK_ATP channels may inhibit apoptosis by preserving mitochondrial membrane potential in neurons, as they do in cardiomyocytes.

In the present study we used H_2O_2 to simulate neuronal apoptosis induced by oxidative stress9 in cultured cerebellar granule neurons, cells that depend on high K⁺ for survival.10

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.

Primary Culture of Cerebellar Granule Neurons

Cerebellar granule neurons were prepared from 7- to 8-day-old Sprague-Dawley rats as previously described.11 Cells were kept for at least 7 days in culture to differentiate.

Experimental Protocol

Cultured cerebellar granule neurons were randomly assigned to 1 of 4 groups, as follows: (1) control; (2) incubation with H_2O_2 (30
H$_2$O$_2$ application. In the experiments with mitochondrial permeability transition pore (PTP) inhibition, 0.2 or 1 μmol/L diazoxide and 500 μmol/L 5HD with H$_2$O$_2$ were used in each experiment to induce the similar effects on the cells in different conditions.

**Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End-Labeling Staining**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed 16 hours after H$_2$O$_2$ exposure (or at the equivalent time in controls) according to the manufacturer’s protocol (Roche), as previously described.$^3$

**Caspase-3 Activity Assay**

Caspase-3 activity was measured by detection of the cleavage of a colorimetric caspase-3 substrate, N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline (DEVD-pNA), with the use of an assay kit. ApoAlert CPP32 (Clontech). The assay was performed according to the manufacturer’s protocol (Roche), as previously described.$^3$

**Flow Cytometry Analysis of Mitochondrial Membrane Potential**

Loss of mitochondrial membrane potential (ΔΨ$_m$) was assessed by a fluorescent indicator, tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) (100 nmol/L), with the use of flow cytometry. Cells were treated with drugs for 120 minutes at 37°C and analyzed by FACSScan (20 000 cells per sample) (Becton Dickinson). Data were analyzed with the use of WinMDI analysis software (http://facs.scripps.edu).

**Time-Lapse Analysis of ΔΨ$_m$ Loss**

Cells were placed in Hank’s salt-based MEM with 25 mmol/L HEPES buffer to avoid pH change in non-CO$_2$ equilibrated environment. After 30-minute incubation in medium containing 100 nmol/L TMRE at 37°C, time-lapse scanning was started with a 20× objective lens. During the scanning, cells were maintained at 37°C with a heater platform installed on the microscope stage. Thirty cells were randomly and prospectively selected in each scan, and TMRE fluorescence intensity was sequentially monitored every 5 minutes.

**Assays for Extracellular Glutamate Concentration**

Glutamate concentrations in the culture medium from each experimental group were assayed by high-performance liquid chromatography with electrochemical detection, as described previously.$^{12}$

**Electrophysiology**

Electrophysiological recordings were performed at room temperature with the whole-cell patch clamp technique. Pipette electrodes had final tip resistances of 5 to 7 mol/L. The internal pipette solution contained the following (mmol/L): potassium glutamate 120, KCl 25, ATP (magnesium salt) 1, EGTA 10, MgCl$_2$ 0.5, HEPES 10 [pH 7.2]. The external bath solution contained the following (mmol/L): NaCl 140, KCl 5, MgCl$_2$ 1, CaCl$_2$ 1, HEPES 10 [pH 7.4]. Designated amounts of drugs were added to the bath when required. Currents were acquired and analyzed with custom-written software.

**Statistical Analysis**

All quantitative data are presented as mean±SEM. Multiple comparisons among groups were performed by 1-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**

**Effects of Diazoxide on Apoptosis in Cerebellar Granule Neurons**

Morphology of cultured cells was assessed by transmitted phase-contrast microscopy (Figure 1A). The control group showed round cell bodies with clear edges and fine dendritic...
network. Incubation with 20 μmol/L H2O2 for 16 hours decreased the number of cells and induced shrinkage of cell bodies and disruption of dendritic networks. Diazoxide mitigated the morphological manifestations of cell damage, and these protective effects were abolished by 5HD. Next we performed TUNEL staining as an indicator of apoptosis. Figure 1B shows representative staining in each group, and Figure 1C shows quantitative results. Control cells showed few TUNEL-positive nuclei (3.6±0.84%). The number of TUNEL-positive nuclei was increased to 47.7±4.7% in the cells exposed to H2O2. Diazoxide significantly decreased the number of TUNEL-positive nuclei to 7.7±2.7%. Treatment with 5HD partially abolished these protective effects of diazoxide (27.5±2.1%).

Effects of Diazoxide on Caspase-3 Activity in Cerebellar Granule Neurons
Since activation of apoptosis-effector caspases such as caspase-3 plays an important role in neuronal cell death after cerebral ischemia,13 we next assayed caspase-3 activity. Figure 1D shows relative caspase-3 activity in each group at various times after H2O2 application. At 8 and 16 hours, caspase-3 activity was significantly increased in the H2O2 group (124% and 161% of control group, respectively), and diazoxide suppressed these increases. 5HD completely abolished the effects of diazoxide.

Effects of Diazoxide on ΔΨm
Dissipation of ΔΨm is a critical event early in the process of apoptosis.14 To examine whether preservation of ΔΨm is associated with the antiapoptotic effect of mitoKATP channel activation, we assessed the effect of diazoxide on ΔΨm using flow cytometry. Incubation with 20 μmol/L H2O2 for 2 hours decreased TMRE fluorescence and shifted the distribution curve leftward to a level similar to that obtained with the cells treated with mitochondrial uncoupler carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP), indicating the depolarization of ΔΨm (Figure 2A). Diazoxide remarkably decreased the fraction of neurons that underwent H2O2-induced dissipation of ΔΨm as shown by the preservation of cells with normal ΔΨm (Figure 2A). 5HD abolished the salutary effect of diazoxide, reverting the distribution to that of the H2O2 group (Figure 2A). Quantitative summary data from flow cytometry are shown in Figure 2B; here, we evaluated the percentage of cells that exhibited a high level of TMRE fluorescence (defined as >102 of fluorescence intensity). Diazoxide inhibited the ΔΨm loss in a concentration-dependent manner, and this effect reached saturation at a concentration of 100 μmol/L (Figure 2B, left). Furthermore, pinacidil, which activates both mitochondrial and sarcolemmal KATP channels,2 also prevented ΔΨm loss in a 5HD-inhibitable manner (Figure 2B, left). Diazoxide or 5HD alone did not measurably affect TMRE fluorescence (Figure 2B, right).

Time-Lapse Analysis of ΔΨm Loss
To examine time-dependent changes of ΔΨm on a single-cell basis, confocal microscopy was performed with the use of cells loaded with TMRE. Figure 3A shows sequential images of TMRE fluorescence in each group. Figure 3C demonstrates time course of TMRE fluorescence in 30 cells randomly and prospectively selected in each group. TMRE fluorescence did not change during 90 minutes of scanning in the control group. In cells treated with H2O2, fluorescence intensity began to decrease within 10 minutes of H2O2 application and progressively decreased thereafter. Diazoxide prevented ΔΨm loss in the majority of cells and delayed the onset of mitochondrial depolarization in those cells that were not fully protected. These effects of diazoxide were cancelled by 5HD. The averaged intensity data from 30 cells are shown in Figure 3C.

Effects of CsA and BA on ΔΨm
PTP is a nonspecific large-conductance channel in the inner mitochondrial membrane. Opening of PTP dissipates ΔΨm by
increasing inner membrane permeability.\textsuperscript{15} We next addressed the question of whether PTP may account for the $\Delta \Psi_m$ dissipation induced by H$_2$O$_2$ in cerebellar granule neurons. The effects of CsA, a PTP inhibitor, on $\Delta \Psi_m$ loss by H$_2$O$_2$ were investigated with flow cytometry (Figure 4A) as well as time-lapse confocal microscopy (Figure 4B) of cerebellar neurons. Incubation with 20 $\mu$mol/L H$_2$O$_2$ for 2 hours resulted in $\Delta \Psi_m$ depolarization to a level similar to that with FCCP-treated cells, as shown by the decrease of TMRE fluorescence. CsA remarkably prevented H$_2$O$_2$-induced dissipation of $\Delta \Psi_m$, as shown by the preservation of cells with intact $\Delta \Psi_m$. Observation of fluorescence intensity in individual cells revealed that CsA inhibited H$_2$O$_2$-induced dissipation of $\Delta \Psi_m$ in a manner similar to that of diazoxide, decreasing the number of cells undergoing $\Delta \Psi_m$ depolarization in unprotected cells. Importantly, 5HD did not inhibit the effect of CsA. BA, an inhibitor of adenine nucleotide translocase (a putative component of the PTP), prevented $\Delta \Psi_m$ loss induced by H$_2$O$_2$ in a manner similar to that of CsA, and 5HD did not inhibit this effect of BA. These results indicate the involvement of PTP in $\Delta \Psi_m$ loss induced by H$_2$O$_2$ and may suggest that mitoK$_{\text{ATP}}$ channels work upstream of PTP. Figure 4C shows pooled flow cytometry data. We evaluated the percentage of cells that exhibited a high level of TMRE fluorescence (defined as $>10^2$ of fluorescence intensity). The pooled data confirm the representative results.

**Effects of Diazoxide on Extracellular Glutamate Concentration**

Given the well-recognized role of glutamate in excitotoxicity and neuronal apoptosis,\textsuperscript{16} we assayed extracellular glutamate concentration in each experimental group. H$_2$O$_2$ stimulation significantly increased glutamate concentration in the culture medium, and diazoxide suppressed this increase in a 5HD-inhibitable manner (Figure 5A).

**Effects of Diazoxide on Surface K$_{\text{ATP}}$ Channels**

In some cells, K$_{\text{ATP}}$ channels exist both in the surface membrane and in the inner mitochondrial membrane. If surface K$_{\text{ATP}}$ channels are present in cerebellar granule neurons, they may be activated by diazoxide; the resultant hyperpolarization could underlie the inhibition of glutamate release and the neuroprotective effects. To determine whether the antiapoptotic effects of diazoxide in cerebellar granule neurons are mediated by mitoK$_{\text{ATP}}$ channels rather than surface K$_{\text{ATP}}$ channels, we measured surface membrane current in cultured cerebellar granule neurons. Figure 5B shows that application of the surface K$_{\text{ATP}}$ channel agonist pinacidil\textsuperscript{2} (100 $\mu$mol/L) or its potent derivative F-1075\textsuperscript{17} (100 $\mu$mol/L)
resulted in modest, statistically insignificant ($P > 0.05$) increases in the steady state outward currents. No hint of an increase, however, was observed with diazoxide (100 $\mu$mol/L). Taken collectively, the results indicate that surface $K_{\text{ATP}}$ channels are sparsely distributed in rat cerebellar granule neurons; the few channels that may be present are not sensitive to diazoxide. Thus, the protective effects we have described are not attributable to surface $K_{\text{ATP}}$ channel activation.

**Discussion**

The principal findings of our study are as follows. (1) Diazoxide, a mito$K_{\text{ATP}}$ channel opener, inhibited the apoptotic cascade and alleviated cell damage induced by oxidative stress. (2) Diazoxide and pinacidil prevented $\Delta\Psi_m$ loss induced by oxidative stress in a 5HD-inhibitable manner. (3) CsA or BA, an inhibitor of PTP, prevented $\Delta\Psi_m$ loss induced by oxidative stress, and this effect was not inhibited by 5HD. (4) Diazoxide prevented the increase in the concentration of extracellular glutamate induced by $H_2O_2$ stimulation. These results indicate that mito$K_{\text{ATP}}$ channel openers protect neurons against oxidative stress by preserving $\Delta\Psi_m$. Inhibition of PTP opening likewise suppresses $H_2O_2$-induced apoptosis. Inhibition of glutamate release may be one mechanism contributing to the neuroprotective effects of diazoxide.

Figure 4. Effects of CsA on $\Delta\Psi_m$ in $H_2O_2$-stimulated cells. A, Representative histograms obtained by flow cytometry. Cells were stimulated by 20 $\mu$mol/L $H_2O_2$. FCCP indicates 10 $\mu$mol/L FCCP. B, Time-dependent alterations of TMRE fluorescence in 30 randomly selected cells. Cells were stimulated by 30 $\mu$mol/L $H_2O_2$. C, Quantitative data of flow cytometry. Data are expressed as percentage of cells with high level of TMRE fluorescence. Data are mean ± SEM (n=3 per group). *P<0.01 vs CsA 0 $\mu$mol/L.

Figure 6 shows a schematic diagram of our working hypothesis to explain the neuroprotection by activation of mito$K_{\text{ATP}}$ channels. Opening of PTP is considered to be a critical event in apoptosis and possibly a trigger of cell death in ischemia/reperfusion injury in the brain. When one considers the present results that both CsA and BA prevented $\Delta\Psi_m$ loss induced by $H_2O_2$, PTP opening is closely involved in the process of apoptosis in neurons. In fact, several reports showed that CsA protected the brain against ischemia/reperfusion injury in rodents. Although we did not directly prove the prevention of PTP opening by diazoxide, the present observations that diazoxide and CsA inhibited $\Delta\Psi_m$ loss in a similar manner may indicate that these 2 drugs affect the same pathway. Furthermore, the results that 5HD did not cancel the effects of CsA and BA suggest that mito$K_{\text{ATP}}$ channels presumably come into play upstream of PTP. Diazoxide was reported to partially depolarize $\Delta\Psi_m$ in neuronal cells, which may attenuate $Ca^{2+}$ accumulation in mitochondria by decreasing the driving force for $Ca^{2+}$ uptake. In fact, diazoxide attenuates the accumulation of mitochondrial $Ca^{2+}$ during simulated ischemia/reperfusion injury in cardiomyocytes. Matrix $Ca^{2+}$ overload, in turn, is known to induce opening of PTP. Hence, activation of mito$K_{\text{ATP}}$ channels may prevent PTP opening by reducing matrix $Ca^{2+}$ overload, thereby inhibiting apoptosis. Reactive oxygen spe-
cies (ROS) generation, another important trigger of PTP opening, was reportedly decreased by diazoxide in hippocampal cells and in isolated heart mitochondria. In contrast, diazoxide increased ROS generation to result in cardioprotection. These results suggest the possibility that a moderate increase of ROS by diazoxide may prevent a subsequent large increase of ROS production. As other possible mechanisms, a recent report demonstrated that diazoxide suppressed the cytochrome c release and translocation of Bax and increased Bcl-2 protein level in cultured hippocampal cells.

Glutamate is the major excitatory neurotransmitter in the central nervous system, and, during the postnatal period, glutamate-activated N-methyl-D-aspartate (NMDA) receptors increase Ca influx into cytosol to trigger changes in neuronal metabolism and gene expression that are necessary for brain development. In contrast, overstimulation of NMDA receptors by glutamate induces cell death in a process known as excitotoxicity, and mitochondrial Ca overload is closely associated with this process. Several studies have shown that extracellular glutamate increases in brain injury, although the precise mechanisms are still controversial. Reversed operation of the glutamate transporter is the main candidate for the increased extracellular glutamate in ischemia, and it may be induced by ATP depletion. In the present study we observed that H2O2 stimulation increased extracellular glutamate concentration, and diazoxide inhibited the increase of glutamate. These results suggest the possibility that reduced glutamate release may be one mechanism of neuroprotection by diazoxide, although the precise mechanism by which activation of mitoKATP channels prevents glutamate release remains unclear. As one possibility, diazoxide may prevent glutamate release by preserving ATP production. However, given the protective effects of diazoxide in cardiomyocytes, suppression of glutamate release may not be the primary mechanism of protection in neuronal cells. We cannot exclude the possibility that increased extracellular glutamate might simply be due to glutamate leakage from dead cells, such that the decrease in glutamate by diazoxide may be the result of (rather than the cause of) reduced cell injury. Further investigation will be needed to elucidate precisely how the suppression of glutamate release is associated with neuroprotection by mitoKATP channel openers.

The accumulation of TMRE in mitochondria depends not only on ΔΨm, but also on the cytosolic concentration of the probe. To exclude the possibility that the effects of diazoxide are mediated by the inhibition of multidrug resistance pump in plasma membrane, we examined the effect of MK 571, a specific multidrug resistance pump inhibitor, on ΔΨm in H2O2-stimulated cells. TMRE fluorescence did not change in MK 571–treated cells, indicating that the ΔΨm-preserving effects of diazoxide or CsA do not depend on multidrug resistance inhibition (data not shown).

Many excitable cells, including some neurons, contain 2 distinct KATP channels: the classic one in the cell membrane (surface KATP channel) and the other in the mitochondrial inner membrane (mitoKATP channel). These 2 channels are known to have important pharmacological differences in the heart. Surface KATP channels from cardiac sarcolemma are resistant to diazoxide and 5HD, whereas mitoKATP channels are sensitive to both agents. There has been much debate as to whether surface KATP channels or mitoKATP channels predominantly contribute to cell protection mainly in the
heart, and the subject is still controversial. A newly identified mitoKATP channel opener, BMS 191095, has cardioprotective effects without activating surface KATP channels, supporting the predominance of mitoKATP channels in the protective effects. In the present study we confirmed that a neuroprotective concentration of diazoxide did not activate surface KATP channels in cultured cerebellar granule neurons. These results indicate that the neuroprotective effects of diazoxide are due to activation of mitoKATP channels, at least in cerebellar granule cells.

When it is considered that apoptosis is a significant contributor to ischemia/reperfusion injury in the brain, 4,8 mitoKATP channels could be a possible target for neuroprotection. Further studies are required to explore more detailed mechanisms of the observed effects.

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