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\textsubscript{ATP}) channels are present in the brain, and several reports have shown that mitoK\textsubscript{ATP} channel openers protect the brain against ischemic injury. However, the precise mechanisms of this protection are not well established. We hypothesized that mitoK\textsubscript{ATP} channel openers prevent apoptosis by preserving mitochondrial membrane potential.

Methods—We investigated the effect of mitoK\textsubscript{ATP} channel openers on apoptosis induced by oxidative stress using cultured cerebellar granule neurons.

Results—The mitoK\textsubscript{ATP} channel opener diazoxide (100 \mumol/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 \mumol/L H\textsubscript{2}O\textsubscript{2}. Diazoxide and another opener, pinacidil, prevented the loss of mitochondrial inner membrane potential (\Delta\Psi\textsubscript{m}) induced by H\textsubscript{2}O\textsubscript{2}. These effects were abolished by 5-hydroxydecanoate (500 \mumol/L), a mitoK\textsubscript{ATP} channel blocker. Cyclosporin A and bongkrekic acid, inhibitors of the mitochondrial permeability transition pore, also prevented \Delta\Psi\textsubscript{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\textsubscript{2}O\textsubscript{2}, but this effect was not attributable to activation of surface K\textsubscript{ATP} channels.

Conclusions—MitoK\textsubscript{ATP} channel openers inhibited apoptosis by preserving mitochondrial inner membrane potential. These beneficial effects may suggest a possible new target for neuroprotection.

Key Words: apoptosis  mitoK\textsubscript{ATP}  neurons  oxidative stress  potassium channels
μmol/L for time-lapse confocal microscopy, and 20 μmol/L for the other experiments). (3) 100 μmol/L diazoxide with H₂O₂; and (4) 100 μmol/L diazoxide and 500 μmol/L 5HD with H₂O₂. In the appropriate groups, diazoxide and 5HD were applied 20 minutes before H₂O₂ application. In the experiments with mitochondrial permeability transition pore (PTP) inhibition, 0.2 or 1 μmol/L cyclosporin A (CsA) or 50 μmol/L bongkrekic acid (BA) was used instead of diazoxide. Appropriate H₂O₂ concentrations and the length of stimulation 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 H₂O₂ 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-nitroanilide (DEVD-pNA), with the use of an assay kit, ApoAlert CPP32 (Clontech). The assay was performed according to the manufacturer’s protocol at 4, 8, and 16 hours of stimulation.

Flow Cytometry Analysis of Mitochondrial Membrane Potential
Loss of mitochondrial membrane potential (ΔΨm) 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 ΔΨm Loss
Cells were placed in Hank’s salt-based MEM with 25 mmol/L HEPES buffer to avoid pH change in non-CO₂ 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, MgCl₂ 0.5, HEPES 10 [pH 7.2]. The external bath solution contained the following (mmol/L): NaCl 140, KCl 5, MgCl₂ 1, CaCl₂ 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 preformed 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 mitoK<sub>ATP</sub> 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 >10<sup>2</sup> 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 sarcosomal K<sub>ATP</sub> 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 3B.

**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. We next addressed the question of whether PTP may account for the \( \Delta \Psi_m \) dissipation induced by \( \text{H}_2\text{O}_2 \) in cerebellar granule neurons. The effects of CsA, a PTP inhibitor, on \( \Delta \Psi_m \) loss by \( \text{H}_2\text{O}_2 \) were investigated with flow cytometry (Figure 4A) as well as time-lapse confocal microscopy (Figure 4B) of cerebellar neurons. Incubation with 20 \( \mu\text{mol/L} \) \( \text{H}_2\text{O}_2 \) for 2 hours resulted in \( \Delta \Psi_m \) depolarization to a level similar to that of with FCCP-treated cells, as shown by the decrease of TMRE fluorescence. CsA remarkably prevented \( \text{H}_2\text{O}_2 \)-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 \( \text{H}_2\text{O}_2 \)-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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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, we assayed extracellular glutamate concentration in each experimental group. \( \text{H}_2\text{O}_2 \) 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 (100 \( \mu\text{mol/L} \)) or its potent derivative P-1075 (100 \( \mu\text{mol/L} \))
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 μmol/L). Taken collectively, the results indicate that surface K$_{ATP}$ 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$_{ATP}$ channel activation.

**Discussion**

The principal findings of our study are as follows. (1) Diazoxide, a mitoK$_{ATP}$ channel opener, inhibited the apoptotic cascade and alleviated cell damage induced by oxidative stress. (2) Diazoxide and pinacidil prevented ΔΨ$_{m}$ loss induced by oxidative stress in a 5HD-inhibitable manner. (3) CsA or BA, an inhibitor of PTP, prevented ΔΨ$_{m}$ 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$_2$O$_2$ stimulation. These results indicate that mitoK$_{ATP}$ channel openers protect neurons against oxidative stress by preserving ΔΨ$_{m}$. Inhibition of PTP opening likewise suppresses H$_2$O$_2$-induced apoptosis. Inhibition of glutamate release may be one mechanism contributing to the neuroprotective effects of diazoxide.

Figure 6 shows a schematic diagram of our working hypothesis to explain the neuroprotection by activation of mitoK$_{ATP}$ channels. Opening of PTP is considered to be a critical event in apoptosis$^{14,18}$ and possibly a trigger of cell death in ischemia/reperfusion injury in the brain.$^{19}$ When one considers the present results that both CsA and BA prevented ΔΨ$_{m}$ loss induced by H$_2$O$_2$, 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.$^{20,21}$ Although we did not directly prove the prevention of PTP opening by diazoxide, the present observations that diazoxide and CsA inhibited ΔΨ$_{m}$ 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 mitoK$_{ATP}$ channels presumably come into play upstream of PTP. Diazoxide was reported to partially depolarize ΔΨ$_{m}$ in neuronal cells,$^{22}$ 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.$^{23}$ Matrix Ca$^{2+}$ overload, in turn, is known to induce opening of PTP.$^{24}$ Hence, activation of mitoK$_{ATP}$ 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\(^{25}\) and in isolated heart mitochondria.\(^{26}\) In contrast, diazoxide increased ROS generation to result in cardioprotection.\(^{27}\) 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.\(^{8}\)

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 \(\text{Ca}^{2+}\) 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,\(^{16}\) and mitochondrial \(\text{Ca}^{2+}\) overload is closely associated with this process.\(^{16}\) Several studies have shown that extracellular glutamate increases in brain injury,\(^{28,29}\) although the precise mechanisms are still controversial. Reversed operation of the glutamate transporter is the main candidate for the increased extracellular glutamate in ischemia,\(^{30}\) and it may be induced by ATP depletion.\(^{31}\) In the present study we observed that \(\text{H}_2\text{O}_2\) 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 mitoK\(_{\text{ATP}}\) channels prevents glutamate release remains unclear. As one possibility, diazoxide may prevent glutamate release by preserving ATP production.\(^{32}\) However, given the protective effects of diazoxide in cardiomyocytes,\(^{3} \) 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 mitoK\(_{\text{ATP}}\) channel openers.

The accumulation of TMRE in mitochondria depends not only on \(\Delta \Psi_m\), 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,\(^{33}\) a specific multidrug resistance pump inhibitor, on \(\Delta \Psi_m\) in \(\text{H}_2\text{O}_2\)-stimulated cells. TMRE fluorescence did not change in MK 571–treated cells, indicating that the \(\Delta \Psi_m\)-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 \(K_{\text{ATP}}\) channels: the classic one in the cell membrane (surface \(K_{\text{ATP}}\) channel)\(^{34}\) and the other in the mitochondrial inner membrane (mito\(K_{\text{ATP}}\) channel).\(^{5}\) These 2 channels are known to have important pharmacological differences in the heart. Surface \(K_{\text{ATP}}\) channels from cardiac sarcolemma are resistant to diazoxide and 5HD, whereas mito\(K_{\text{ATP}}\) channels are sensitive to both agents.\(^{2}\) There has been much debate as to whether surface \(K_{\text{ATP}}\) channels or mito\(K_{\text{ATP}}\) channels predominantly contribute to cell protection mainly in the

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**Figure 5.** A, Effects of diazoxide on extracellular glutamate concentration. Data are relative percentage of glutamate concentration to control. CON indicates control; \(\text{H}_2\text{O}_2, 20 \mu\text{mol/L H}_2\text{O}_2\); DZ, \(\text{H}_2\text{O}_2+100 \mu\text{mol/L diazoxide}\); and 5HD, \(\text{H}_2\text{O}_2+\text{diazoxide}+500 \mu\text{mol/L 5HD}\). Data are mean±SEM (n=4 per group). *P<0.05 vs CON. B, Distribution of raw (open squares) and averaged (solid circles) steady state outward current densities measured at 300 ms after depolarizing cells to +20 mV from a holding potential of −80 mV preceded by a 100-ms prepulse to 0 mV in the absence and presence of 100 μmol/L pinacidil (Pina), 100 μmol/L P-1075, and 100 μmol/L diazoxide (DZ) as indicated.

**Figure 6.** Proposed mechanism of neuroprotection by activation of mito\(K_{\text{ATP}}\) channels.
heart, and the subject is still controversial. A newly identified mitoK<sub>ATP</sub> channel opener, BMS 191095, has cardioprotective effects without activating surface K<sub>ATP</sub> channels, supporting the predominance of mitoK<sub>ATP</sub> channels in the protective effects. In the present study we confirmed that a neuroprotective concentration of diazoxide did not activate surface K<sub>ATP</sub> channels in cultured cerebellar granule neurons. These results indicate that the neuroprotective effects of diazoxide are due to activation of mitoK<sub>ATP</sub> channels, at least in cerebellar granule cells.

When it is considered that apoptosis is a significant contributor to ischemia/reperfusion injury in the brain, 1,4,8 mitoK<sub>ATP</sub> 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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