Adenosine Primes the Opening of Mitochondrial ATP-Sensitive Potassium Channels
A Key Step in Ischemic Preconditioning?

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Background—Adenosine can initiate ischemic preconditioning, and mitochondrial ATP-sensitive potassium (K\textsubscript{ATP}) channels have emerged as the likely effectors. We sought to determine the mechanistic interactions between these 2 observations.

Methods and Results—The mitochondrial flavoprotein oxidation induced by diazoxide (100 \(\mu\)mol/L) was used to quantify mitochondrial K\textsubscript{ATP} channel activity in intact rabbit ventricular myocytes. Adenosine (100 \(\mu\)mol/L) increased mitochondrial K\textsubscript{ATP} channel activity and abbreviated the latency to mitochondrial K\textsubscript{ATP} channel opening. These potentiating effects were entirely prevented by the adenosine receptor antagonist 8-((p-sulfophenyl)-theophylline (100 \(\mu\)mol/L) or by the protein kinase C inhibitor polymyxin B (50 \(\mu\)mol/L). The effects of adenosine and diazoxide reflected mitochondrial K\textsubscript{ATP} channel activation, because they could be blocked by the mitochondrial K\textsubscript{ATP} channel blocker 5-hydroxydecanoate (500 \(\mu\)mol/L). In a cellular model of simulated ischemia, adenosine mitigated cell injury; this cardioprotective effect was blocked by 5-hydroxydecanoate but not by the surface-selective K\textsubscript{ATP} channel blocker HMR1098. Moreover, adenosine augmented the cardioprotective effect of diazoxide. A quantitative model of mitochondrial K\textsubscript{ATP} channel gating reproduced the major experimental findings.

Conclusions—Our results support the hypothesis that adenosine receptor activation primes the opening of mitochondrial K\textsubscript{ATP} channels in a protein kinase C-dependent manner. The findings provide tangible links among various key elements in the preconditioning cascade. (Circulation. 2000;102:800-805.)

Key Words: adenosine ■ ischemia ■ ion channels ■ mitochondria

Ischemic preconditioning (IPC) is the endogenous mechanism whereby brief periods of ischemia paradoxically protect the myocardium against the damaging effects of subsequent prolonged ischemia.\(^1\) IPC exists in all species examined, including humans.\(^2\)-\(^4\) Although the precise cellular mechanisms responsible for IPC remain elusive, adenosine released by the ischemic heart has been reported to initiate IPC.\(^5\) Protein kinase C (PKC) features prominently in the signal transduction pathway between adenosine A\(_1\) receptors and the final effector.\(^6\) Adenosine and PKC have been shown to activate ATP-sensitive potassium (K\textsubscript{ATP}) channels in cardiac myocytes.\(^7\)-\(^9\) Consistent with the idea that K\textsubscript{ATP} channels may be the end effectors of IPC, stimulation of adenosine A\(_1\) receptors mimics IPC, and this effect is abolished by the K\textsubscript{ATP} channel inhibitor glibenclamide.\(^10\)

The cardioprotective effects were initially attributed to sarcolemmal K\textsubscript{ATP} (surfaceK\textsubscript{ATP}) channels.\(^11\),\(^12\) However, recent studies provide evidence that mitochondrial K\textsubscript{ATP} (mitoK\textsubscript{ATP}) channels are the dominant players. Diazoxide, a selective mitoK\textsubscript{ATP} channel opener in cardiac cells,\(^13\) protects rabbit ventricular myocytes in a pelleting model of ischemia,\(^14\) improves functional recovery after ischemia in Langendorff-perfused rat and rabbit hearts,\(^15\) and reduces infarct size in rabbit hearts.\(^16\) On the other hand, the selective mitoK\textsubscript{ATP} channel blocker 5-hydroxydecanoate (5HD)\(^17\) antagonizes diazoxide-induced cardioprotection\(^14,15\) and abolishes genuine IPC.\(^16\),\(^18\)-\(^20\) Furthermore, PKC activation potentiates mitoK\textsubscript{ATP} channel opening in rabbit ventricular myocytes.\(^17\)

Accordingly, the present study was designed to determine whether adenosine modulates mitoK\textsubscript{ATP} channels. Our results demonstrate that adenosine potentiates mitoK\textsubscript{ATP} channel activation via a PKC-mediated pathway and protects the myocardium from ischemia.

Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985).
Preparation of Rabbit Myocytes

Rabbit ventricular myocytes were isolated by conventional enzymatic dissociation, as described previously. In brief, hearts were excised from anesthetized (30 mg/kg pentobarbital IV) New Zealand White rabbits (weighing 1 to 2 kg) and mounted on a Langendorff apparatus. The heart was perfused with modified Krebs-Henseleit solution composed of (mmol/L) NaCl 119, KCl 5, NaHCO3 25, KH2PO4 1, MgSO4 1, CaCl2 2, and glucose 10. The perfusate was bubbled with 95% O2/5% CO2 and maintained at 37°C. After 5 minutes of perfusion, hearts were perfused without Ca2+ for another 5 minutes, after which the perfusion solution was switched to one containing collagenase (0.8 mg/mL, Worthington type II). The perfusion pressure was monitored, and the flow rate was adjusted to maintain perfusion pressure at ~75 mm Hg. After 25 to 30 minutes of collagenase perfusion, hearts were removed from the perfusion apparatus, and the atria were trimmed away. The ventricles were minced and incubated in a shaking bath for another 5 minutes in collagenase-containing solution. Cells were then filtered through nylon mesh and washed several times with Ca2+-free solution. Calcium concentration was gradually brought back to 1 mmol/L.

Flavoprotein Fluorescence

Opening of mitoKATP channels dissipates the inner mitochondrial membrane potential established by the proton pump. This dissipation accelerates electron transfer by the respiratory chain and, if uncompensated by increased production of electron donors, leads to net oxidation of the mitochondria. Therefore, the mitochondrial redox state was monitored by measuring the autofluorescence of flavin adenine dinucleotide–linked enzymes in the mitochondria, as described by Liu et al. After isolation, cells were cultured on laminin-coated coverslips in medium 199 with 5% FBS at 37°C. Experiments were performed the next day. Cells were placed in a recording chamber and superfused with external solution containing (mmol/L) NaCl 140, KCl 5, MgCl2 1, CaCl2 1, and HEPES 10 (pH 7.4 with NaOH) at room temperature (~22°C). Endogenous flavoprotein fluorescence was excited for 100 ms every 6 seconds by use of a xenon arc lamp with a band-pass filter centered at 480 nm. Emitted fluorescence was recorded at 530 nm by a photomultiplier tube and digitized. The redox signal was averaged during the excitation window and calibrated with the values after exposure to 2,4-dinitrophenol (DNP), which uncouples respiration from ATP synthesis, collapses the mitochondrial potential, and induces maximal oxidation. Therefore, the values of flavoprotein fluorescence were expressed as a percentage of the DNP-induced fluorescence. By focusing on individual myocytes with a ×40 objective, fluorescence was monitored from one cell at a time.

Cellular Model of Simulated Ischemia

A cellular model of simulated ischemia modified from Vander Heide et al. was used to quantify cell injury. In brief, cells were washed with incubation buffer containing (mmol/L) NaCl 119, NaHCO3 25, KH2PO4 1.2, KCl 4.8, MgSO4 1.2, CaCl2 1, HEPES 10, glucose 11, creatine 24.9, and taunine 58.5 and supplemented with 1% basal medium eagle amino acids and 1% minimum essential medium nonessential amino acids (pH 7.4 with NaOH). An aliquot of each cell suspension (0.5 mL) was placed into a microcentrifuge tube and centrifuged for 15 seconds into a pellet. Approximately 0.25 mL of excess supernatant was removed to leave a thin fluid layer above the pellet, and 0.2 mL of mineral oil was layered on the top of the pellet to prevent gaseous diffusion. After 60 and 120 minutes of simulated ischemia, 5 μL of cell pellet was sampled through the oil layer and mixed with 75 μL of 85 mOsm/L hypotonic staining solution containing (mmol/L) NaHCO3 11.9, KH2PO4 0.4, KCl 2.7, MgSO4 0.8, and CaCl2 1, along with 0.5% glutaraldehyde and 0.5% trypan blue. Microscopic examination was performed 2 to 5 minutes after mixing to determine the permeability of the cells to trypan blue. Cells permeable to trypan blue were counted as stained (ie, irreversibly injured) and expressed as a percentage of the total cells counted (>300 for each sample). In the control group, cells were pelleted and sampled at 60 and 120 minutes. For the adenosine-treated group, adenosine at a concentration of 100 μmol/L was added to the solution 15 minutes before the pelleting. Cells treated with adenosine in the presence of 500 μmol/L SHD or in the presence of 30 μmol/L HMR1098 were likewise pelleted and sampled. In another series of experiments, cells exposed to various concentrations of diazoxide with or without 100 μmol/L adenosine were pelleted and sampled after 60 minutes of simulated ischemia. Once applied, drugs were not washed out and were present throughout the period of simulated ischemia. Experiments were performed at 37°C. Individual experiments in each group were performed on cells isolated from different hearts.

Chemicals

Collagenase (type II) was purchased from Worthington. Adenosine, diazoxide, 8-(p-sulfophenyl)-theophylline (SPT), polymyxin B, and DNP were obtained from Sigma Chemical Co. Sodium 5HD was purchased from Research Biochemicals International. HMR1098 was a gift from Hoechst Marion Roussel Chemical Research (Frankfurt, Germany). Adenosine, diazoxide, and SPT were dissolved in dimethyl sulfoxide before being added into experimental solutions. The final concentration of dimethyl sulfoxide was <0.1%.

Quantitative Modeling of Channel Function

To rationalize the opening of mitoKATP channels underlying flavoprotein oxidation, we implemented Markov gating models of the Hodgkin-Huxley type (Figure 7B). As a first approximation, we assumed that flavoprotein oxidation parallels mitoKATP channel opening. Simulations were executed by use of Origin (Microcal Software) on a personal computer (Figure 7C and 7D).

Data Analysis

Data are presented as mean±SEM, and the number of cells or experiments is shown as n. Cell pelleting data were analyzed by ANOVA combined with the Fisher post hoc test, and fluorescence data were analyzed by the Student t test. A value of P<0.05 was considered significant.

Results

The effects of adenosine on mitoKATP channels were examined by measuring flavoprotein oxidation elicited by diazoxide. Figure 1A shows representative recordings of flavoprotein fluorescence in a cell exposed twice to diazoxide. Diazoxide (100 μmol/L) reversibly oxidized flavoproteins via the opening of mitoKATP channels. Subsequent exposure to adenosine (100 μmol/L) alone had no effects on flavoprotein fluorescence, but a second application of diazoxide in the continued presence of adenosine increased flavoprotein fluorescence above the levels reached in the first application. Note also that in the presence of adenosine, the second application of diazoxide elicited oxidation more quickly than the first. As summarized in Figure 1B, diazoxide (100 μmol/L) reversibly increased flavoprotein oxidation to 42±5% of the DNP value (n=5). Adenosine (100 μmol/L) significantly increased the diazoxide-induced flavoprotein oxidation to 57±4% of the DNP value (n=5, P<0.01).

Figure 1C summarizes the latency to mitoKATP activation, measured as the time required to increase flavoprotein oxidation to 20% of maximal after application of diazoxide. The exposure to diazoxide in the presence of adenosine abbreviated the latency from 6.6±1.1 to 4.3±0.5 minutes (n=5, P<0.05). Repeated applications of diazoxide alone neither potentiate nor accelerate flavoprotein oxidation.

To confirm that adenosine potentiated and accelerated the oxidative effect of diazoxide via stimulation of adenosine A1...
receptors, we examined the effects of the adenosine A1 receptor antagonist SPT. When 100 μmol/L SPT was present in the perfusate, adenosine (100 μmol/L) failed to augment the oxidative effect of diazoxide and did not abbreviate the latency to mitoKATP activation (Figure 2A). As summarized in Figure 2B, in the presence of SPT (100 μmol/L) with

![Figure 1. Effects of adenosine (ADO) on diazoxide (DIAZO)-induced flavoprotein oxidation. A, Time course of flavoprotein fluorescence in cell exposed to DIAZO (100 μmol/L) and ADO (100 μmol/L). Flavoprotein fluorescence was calibrated by exposing cells to DNP (100 μmol/L) at the end of experiments. Bars indicate periods when cells were exposed to each drug. B, Summarized data for percentage of DIAZO-induced flavoprotein oxidation measured in the absence (DIAZO) and presence of adenosine (DIAZO+ADO). **P<0.01 vs DIAZO group. C, Summarized data for time required to activate mitoKATP channel (latency). DIAZO(1) and ADO+DIAZO(2) indicate first exposure to DIAZO and second exposure to DIAZO in the presence of ADO, respectively (n=5). *P<0.05 vs DIAZO(1) group.](http://circ.ahajournals.org/content/circulation/102/6/802/F1.large.jpg)

Figure 2. Effects of ADO and SPT on DIAZO-induced flavoprotein oxidation. A, Time course of flavoprotein fluorescence. Bar indicates periods when cells were exposed to DIAZO (100 μmol/L), ADO (100 μmol/L), and SPT (100 μmol/L). B, Summarized data for percentage of flavoprotein oxidation. C, Summarized data for the latency to mitoKATP activation. DIAZO(1) and SPT+ADO+DIAZO(2) indicate first exposure to DIAZO and second exposure to DIAZO in the presence of ADO and SPT, respectively (n=4).

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Figure 3. Effects of ADO and polymyxin B (PMB) on DIAZO-induced flavoprotein oxidation. A, Time course of flavoprotein fluorescence. Bar indicates periods when cells were exposed to DIAZO (100 μmol/L), ADO (100 μmol/L), and PMB (50 μmol/L). B, Summarized data for percentage of flavoprotein oxidation. C, Summarized data for the latency to mitoKATP activation. DIAZO(1) and PMB+ADO+DIAZO(2) indicate first exposure to DIAZO and second exposure to DIAZO in the presence of ADO and PMB, respectively (n=5).

![Figure 3. Effects of ADO and polymyxin B (PMB) on DIAZO-induced flavoprotein oxidation. A, Time course of flavoprotein fluorescence. Bar indicates periods when cells were exposed to DIAZO (100 μmol/L), ADO (100 μmol/L), and PMB (50 μmol/L). B, Summarized data for percentage of flavoprotein oxidation. C, Summarized data for the latency to mitoKATP activation. DIAZO(1) and PMB+ADO+DIAZO(2) indicate first exposure to DIAZO and second exposure to DIAZO in the presence of ADO and PMB, respectively (n=5).](http://circ.ahajournals.org/content/circulation/102/6/802/F3.large.jpg)

adenosine (100 μmol/L), diazoxide increased flavoprotein oxidation to 38±9% of the DNP value (n=4). This degree of oxidation was comparable to that achieved by exposure to diazoxide alone (41±6% of the DNP value, n=4). Furthermore, the latency to mitoKATP activation was not abbreviated when the adenosine was co-applied with SPT (6.8±1.3 versus 6.5±1.4 minutes, n=4, P=NS) (Figure 2C). We also examined whether activation of endogenous PKC by adenosine results in mitoKATP channel activation. Chelerythrine and calphostin C, both of which are reported to be more selective PKC inhibitors, enhanced the emitted fluorescence signal nonspecifically. Therefore, polymyxin B was used to inhibit endogenous PKC in the present experiments. As shown in Figure 3, in the presence of polymyxin B (50 μmol/L), adenosine (100 μmol/L) did not alter the oxidative effect of diazoxide (43±3% versus 42±2% of the DNP value, n=5, P=NS) and could not abbreviate the latency to mitoKATP activation (7.9±0.9 versus 8.4±0.7 minutes, n=5, P=NS).

To verify that the diazoxide-induced flavoprotein oxidation observed in the presence of adenosine reflects the activation of mitoKATP channels, the effects of the mitoKATP channel blocker 5HD were examined. 5HD (500 μmol/L) suppressed diazoxide-induced flavoprotein oxidation from 39±4% to 9±2% of the DNP value (n=4, P<0.01), even with the concomitant application of adenosine (Figure 4). These results indicate that adenosine primes the opening of mitoKATP channels through the adenosine A1 receptor-mediated activation of PKC.

Our finding that adenosine increases diazoxide-induced mitochondrial oxidation begs the question of whether adenosine potentiates diazoxide-induced cardioprotection. Figure 5 plots the fraction of cells stained by 60 minutes of simulated ischemia as a percentage of the total number of viable cells before ischemia. Diazoxide decreased the percentage of cells...
stained during 60 minutes of ischemia, in a concentration-dependent manner. Inclusion of 100 μmol/L adenosine alone significantly decreased the extent of cell staining during ischemia. In addition, the cardioprotective effects of diazoxide were significantly augmented by simultaneous application of 100 μmol/L adenosine. The cardioprotection in the presence of both diazoxide (100 μmol/L) and adenosine (100 μmol/L) surpassed the level achieved by diazoxide alone (11±1%, n=5).

In the next series of experiments, we tested the idea that mitoKATP channels rather than surfaceKATP channels act as effectors for cardioprotection afforded by adenosine. As shown in Figure 6, simulated ischemia for 60 and 120 minutes stained 37±2% (n=6) and 47±2% (n=4) of cells, respectively (control group). Inclusion of adenosine (100 μmol/L) significantly decreased the cells stained during ischemia to 21±2% (n=6) after 60 minutes and 27±3% (n=4) after 120 minutes of ischemia (adenosine-treated group, P<0.001 versus control group). The cardioprotective effects of adenosine were abolished by 500 μmol/L 5HD (40±4% after 60 minutes and 49±3% after 120 minutes of ischemia). In contrast, the selective surfaceKATP channel inhibitor HMR1098 (30 μmol/L) did not abolish the cardioprotection by adenosine. These results indicate that adenosine primes the opening of mitoKATP channels and thereby protects myocytes against ischemic damage.

**Discussion**

Diazoxide targets mitoKATP but not surfaceKATP channels in heart cells and oxidizes the mitochondrial matrix by opening mitoKATP channels.14 The oxidative effects of diazoxide are reversible and reproducible: under basal conditions, the degree of oxidation is identical during first and second exposures to diazoxide, and there is no significant difference in the latencies during first and second exposures.14,17 In the present study, we found that adenosine potentiated the oxidative effects of diazoxide and abbreviated the latency to mitoKATP channel activation on the application of diazoxide (Figure 1). The results agree qualitatively and quantitatively with those of Sato et al,17 in which PKC-activating phorbol ester (phorbol 12-myristate 13-acetate) augmented and accelerated the mitochondrial oxidation induced by diazoxide. These findings led us to test the idea that PKC is downstream from the adenosine A1 receptor and precedes the activation of mitoKATP channels. Both the A1 receptor antagonist SPT and the PKC inhibitor polymyxin B prevented the ability of adenosine to augment and accelerate the oxidative effect of diazoxide (Figures 2 and 3). Moreover, the mitoKATP channel inhibitor 5HD blocked diazoxide-induced mitochondrial matrix oxidation in the presence of adenosine (Figure 4). These results taken together suggest that adenosine A1 receptors link to mitoKATP channels through PKC.

We have observed 2 classes of effects of agents on mitoKATP channels. The KATP channel opens diazoxide and pinacidil open the channels directly, leading to net mitochondrial oxidation.14,17 In contrast, adenosine and PKC-stimulating phorbol esters act to prime the channels17: instead of opening them directly, these agents potentiate the subsequent opening of mitoKATP channels. Thus, we propose the mechanism depicted schematically in Figure 7A. MitoKATP channels exist in 3 distinct states: resting, primed, and open. “Virgin” myocardium contains only resting channels. Such channels cannot open directly; they must first traverse through several intermediate nonconducting states before...
Figure 7. A, Schematic model of mitoK<sub>ATP</sub> channels. B, Markov models based on Hodgkin-Huxley n<sup>e</sup> (top) and n<sup>o</sup> (bottom) kinetics. C and D, Simulated experimental data for time courses of flavoprotein fluorescence shown in Figure 1, here approximated as channel open probability (P<sub>open</sub>). Both simulations postulate 3-minute latency for drug diffusion from extracellular space to mitochondria. For upper and lower gating schemes in panel B, P<sub>open</sub> values are given by 0.40[1-exp(−t/2.3)]<sup>a</sup> and 0.55[1-exp(−t/1.5)], respectively. The calculated values of α, β, α′, and β′ are 0.34, 0.09, 0.37, and 0.29, respectively.

opening. Diazoxide application to resting channels initiates the opening process, but a delay is introduced by the obligatory transit through intermediate nonconducting states. Adenosine and PKC activators shift mitoK<sub>ATP</sub> channels into the primed conformation, from which they can open in response to subsequent direct activation. Diazoxide readily opens channels that are primed; however, the response in virgin myocardium is slow, because the primed channel state is unpopulated. To test whether the scheme in Figure 7A suffices to reproduce our findings, we created an equivalent Markov model for quantitative simulation (Figure 7B).<sup>22</sup> We assume that virgin channels are all in the C<sub>1</sub> state; exposure to diazoxide initiates channel opening, either along the upper (virgin) trajectory (C<sub>1</sub>→ C<sub>2</sub>→ C<sub>3</sub>→ C<sub>4</sub>→ O) or along the lower (preconditioned) one (C<sup>*</sup><sub>0</sub>→ O<sup>*</sup>). The simulation results in Figure 7C reveal that exposure of virgin myocardium to diazoxide opens the channels with some delay. In contrast, Figure 7D shows that diazoxide opens primed channels briskly and intensely. Note that adenosine itself does not open the channels; it merely shifts them into a primed state from which they can open much more readily.

The sort of mechanism proposed in Figure 7 has close parallels in numerous studies of ion channels. The model itself is a direct adaptation of the classical Hodgkin-Huxley gating scheme for voltage-dependent potassium channels.<sup>22</sup> The proposed regulatory mechanism also has well-established precedents. For example, L-type calcium channels open in response to membrane depolarization, but they do so quite infrequently in the basal “resting” state. cAMP-dependent phosphorylation shifts the channels into “primed” states, from which they open much more vigorously in response to depolarization, but such phosphorylation does not suffice to open the channels.<sup>25,26</sup> Pharmaceutical agonists (eg, Bay K8644, Bayer) can open calcium channels directly in a manner quite analogous to the ability of diazoxide to open mitoK<sub>ATP</sub> channels.<sup>25</sup> Although the model rationalizes the pharmacology and regulation of mitoK<sub>ATP</sub> channels in a biologically plausible manner, we must stress that it is presented primarily for heuristic reasons; the details (eg, the precise number of nonconducting states) are underconstrained and subject to refinement as more data become available.

The model proposed in Figure 7 rationalizes the combined effects of adenosine and diazoxide: cardioprotection would be maximized if both priming and direct activation are operative. Similar reasoning may explain the cardioprotective effects of adenosine itself. Despite the fact that adenosine does not suffice to open mitoK<sub>ATP</sub> channels, it shifts channels into the primed state, from which they can be opened much more readily by endogenous stimuli during ischemia (most likely ATP depletion and/or ADP accumulation,<sup>27</sup> although changes in guanine nucleotides<sup>28</sup> or nitric oxide<sup>29</sup> may also play a role). Thus, adenosine can be cardioprotective without direct channel opening.

The salient novel finding from the present study is that the cardioprotection induced by adenosine can be inhibited by the mitoK<sub>ATP</sub> channel blocker 5HD but not by HMR1098 (Figure 6). Unlike 5HD, HMR1098<sup>30</sup> targets surfaceK<sub>ATP</sub> without suppressing mitoK<sub>ATP</sub> channels; HMR1098 at the concentration used in the present study (30 μmol/L) inhibits surface K<sub>ATP</sub> currents activated by metabolic inhibition and by surfaceK<sub>ATP</sub> channel opener P-1075 but has no effect on mitoK<sub>ATP</sub> channels in rabbit ventricular cells.<sup>31</sup> These results, taken together, indicate that cardioprotective effects of adenosine are mediated by the opening of mitoK<sub>ATP</sub> channels.

How might IPC be explained from the present results? We conjecture that in preconditioned myocardium, adenosine receptor activation primes mitoK<sub>ATP</sub> channels in a PKC-dependent manner. Consequently, rapid and robust opening of mitoK<sub>ATP</sub> channels during lethal ischemia protects myocytes against ischemic damage. In agreement with this notion, Miura et al<sup>32</sup> have reported that the adenosine A<sub>1</sub> receptor agonist R-phenylisopropyl-adenosine mimics the infarct size-limiting effects of IPC; this protection is prevented by the PKC inhibitor calphostin C and the mitoK<sub>ATP</sub> channel inhibitor 5HD. The findings further implicate mitoK<sub>ATP</sub> channels and provide tangible links among various key elements in the preconditioning cascade.

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