Opening of Mitochondrial $K_{\text{ATP}}$ Channels Triggers Cardioprotection
Are Reactive Oxygen Species Involved?

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Ischemic preconditioning, a phenomenon in which brief episodes of ischemia and reperfusion paradoxically protect the heart against subsequent lethal ischemia, has been conceptually divided into triggers and mediators/effectors. The trigger, which acts before the index ischemia, is followed by the protection of mediators/effectors during the lethal ischemia. Known triggers include activation of adenosine receptors, $\alpha_1$-adrenergic receptors and opioid receptors, elevated intracellular Ca$^{2+}$, and increased reactive oxygen species (ROS).

The mitochondrial ATP-dependent potassium channel ($\text{mito}K_{\text{ATP}}$) has been proposed to be the mediator of this protection. The link between the trigger and effector may be the activation of protein kinases (eg, protein kinase C, tyrosine kinase, and downstream kinases), which may phosphorylate mito$K_{\text{ATP}}$ causing the channel to open early and/or to a greater extent to reduce injury during the lethal ischemia. Interestingly, opening of mito$K_{\text{ATP}}$ can also trigger cardioprotection. Hearts treated with the mito$K_{\text{ATP}}$ opener diazoxide for a brief period before ischemia had significantly smaller infarction. The triggering effect from diazoxide can be blocked by protein kinase C and tyrosine kinase inhibitors, suggesting that diazoxide activates protein kinases, acting similarly to other triggers. This effect was lost when ROS scavengers were coadministered with diazoxide. ROS are known to activate protein kinases and act as a trigger.

In this issue of Circulation Research, Forbes et al provide a direct demonstration that opening of mito$K_{\text{ATP}}$ increases ROS production in isolated rat ventricular myocytes. Using a ROS-sensitive fluorescent probe 2',7'-dichlorofluorescin (DCF), they showed that diazoxide as well as pinacidil (a nonselective $K_{\text{ATP}}$ opener) increased DCF fluorescence, implying an elevated ROS production. Furthermore, a selective mito$K_{\text{ATP}}$ blocker 5-hydroxydecanoate (5-HD) abolished the increase. The antioxidants N-acetylcysteine or N-mercaptopropionylglycine also blocked the fluorescence increase. Exposing hearts to diazoxide before ischemia improved functional recovery after 20 minutes of global ischemia in their isolated rat heart model, although a classical trigger effect was not tested, because there was no diazoxide washout period before the ischemia.

Although there is no question that a mild ROS stress can trigger cardioprotection by activating protein kinases, the mechanistic links between mito$K_{\text{ATP}}$ opening and ROS are unknown. Most of the knowledge about mito$K_{\text{ATP}}$ on mitochondrial energetics and function has been gained from studies on isolated mitochondria from heart and liver. A well-described consequence of mito$K_{\text{ATP}}$ opening is matrix swelling. Although some studies have shown that mito$K_{\text{ATP}}$ openers increase mitochondrial respiration, depolarize mitochondrial membrane potential partially, and decrease Ca$^{2+}$ uptake into mitochondria, others have not observed these effects. Studies from Kowaltowski et al have shown no significant changes of respiration, mitochondrial membrane potential, or Ca$^{2+}$ uptake by opening mito$K_{\text{ATP}}$. Although such studies of isolated mitochondria have proven invaluable for assessing the direct effects of compounds on mitochondrial function, there are several considerations to keep in mind when comparing such results with more intact preparations.

First, the choice of medium components is often distinctly unphysiological (eg, the presence of high sucrose, choice of substrates, Ca$^{2+}$ concentration, ionic composition, and ATP concentration), and critical interactions with other cellular components are missing, including the absence of a cytoskeleton (which apparently is critical for diazoxide-induced protection) and the lack of coupling with energy-consuming sites (eg, myofibrils and ion transport ATPases). Furthermore, high-oxygen partial pressures (>159 mm Hg) are typically present that are orders of magnitude greater than the in vivo mitochondrial milieu under normoxic conditions (~2 to 3 mm Hg). Such a hyperoxic condition will undoubtedly increase oxidative stress. An additional problem is introduced when comparing drug concentrations used in isolated mitochondria with intact cells or hearts where the intracellular concentration of the drug is unknown. Furthermore, diazoxide has been shown to affect mitochondrial membrane potential and energy metabolism nonspecifically in pancreatic $\beta$-cells. However, the observations that the nonspecific effect was not blocked by glybenclamide whereas the protective effect of diazoxide was eliminated with glybenclamide and 5-HD and 5-HD also abolished the mitochondrial oxidative effect of diazoxide strongly argue against nonspecific effects of diazoxide in the observed cardioprotection and mitochondrial oxidation.

Intact isolated myocyte studies are one step closer to the intact system but are still subject to some of the limitations.
mentioned above, including choice of external solution, substrate selection, hyperoxia, and an unphysiologically low workload. Nevertheless, the ability to control some of these factors and to monitor cellular ion concentrations and mitochondrial redox balance has enabled us to determine if mitoK<sub>ATP</sub> opening occurs in intact cells. Our group has used the native autofluorescence of mitochondrial flavoproteins to monitor the redox state of the mitochondria and showed that diazoxide (as well as pinacidil and nicorandil) causes a significant partial oxidation of mitochondrial flavoproteins, which can be blocked in a competitive and isoform-selective manner by 5-HD but not the surface K<sub>ATP</sub>-selective antagonist HMR1098, indicating that the response is attributable to mitoK<sub>ATP</sub> opening. The concentration dependence (K<sub>1/2</sub> of 27 μmol/L) and pharmacology of this redox response correlates well with cardioprotection in both perfused hearts<sup>12</sup> and cellular models of ischemia. In the study by Forbes et al., the effect of diazoxide on the cellular autofluorescence was almost undetectable under their recording conditions, but a clear change on diazoxide addition (∼120 seconds in their Figure 1) is still evident. Unfortunately, the low gain of the recording, the high background noise, and a very narrow emission bandpass did not permit a detailed comparison between the ROS response and flavoprotein oxidation. Also not tested were the effects of inhibitors of specific mitochondrial sites on the ROS production. It is worth noting that the partial oxidation of flavoproteins has sometimes been misinterpreted to indicate that the mitochondrial membrane potential is highly depolarized by diazoxide. This is, in fact, not the case. The mitochondrial membrane depolarization associated with diazoxide exposure is likely to be <∼15 mV and difficult to detect in intact myocytes. Whether such a small change in mitochondrial membrane potential could be a factor limiting Ca<sup>2+</sup> accumulation during or after ischemia, as previously proposed, remains to be determined; however, even small changes in the electrochemical driving force could potentially lead to large differences in total mitochondrial Ca<sup>2+</sup> accumulation over long periods of time.

How mitoK<sub>ATP</sub> opening might be linked to ROS production is unclear. It is known that the mitochondrial electron transport chain can be an important source of ROS in isolated myocytes, most likely originating from the cytochrome b-c<sub>1</sub> segment of complex III in the respiratory chain. However, it is not clear whether ROS will be produced from an increase or decrease in flux through the electron transfer chain. Vanden Hoek et al. demonstrated that ROS increased during a period of hypoxia, when electron transfer chain activity was presumably lower because of a lack of oxygen. They attributed this to downstream block of electron transfer leading to shunting of electrons to ROS production. Reperfusion after a brief hypoxia, when mitochondrial respiration should return to normal or even higher, actually reduced ROS production. Other studies have also shown that mitochondrial depolarization and stimulated electron transport chain have no effect or reduce mitochondrial ROS generation in intact cells. Thus, it is difficult to predict whether the opening of the mitoK<sub>ATP</sub> channel, presumably associated with partial uncoupling, should elevate ROS production from mitochondria. There are also no data available on the possible effect of mitochondrial matrix volume expansion on ROS generation, nor is there a clear relationship between changes in mitochondrial redox balance and the state of thiol oxidation in the cell, the site targeted by the antioxidants used in the study by Forbes et al. The effects of opening of mitoK<sub>ATP</sub> are likely to be complex, and these effects may not only affect ROS production from mitochondria but also from nonmitochondrial sources. Additionally complicating the issue is the observation that exogenously generated ROS may trigger a larger release of ROS derived from mitochondria.

Although it has only recently emerged that mitoK<sub>ATP</sub> opening may trigger cardioprotection and such an effect may involve ROS, it has been long known that K<sub>ATP</sub> openers reduce ROS production during reperfusion. Pinacidil applied during reperfusion reduced ROS production and limited cell death, and these effects were eliminated in the presence of 5-HD, indicating a role of mitoK<sub>ATP</sub>. Diazoxide also reduced ROS production during reperfusion. Possible interactions between ROS and mitoK<sub>ATP</sub> have been demonstrated in plant mitochondria. Opening of plant mitoK<sub>ATP</sub> reduced superoxide anion generation, and superoxide anion formation stimulated plant mitoK<sub>ATP</sub>, suggesting a possible feedback mechanism to protect against ROS in plants. Whether a similar feedback mechanism of ROS and mitoK<sub>ATP</sub> also exists in mammalian mitochondria is unknown. If elevation of ROS by mitoK<sub>ATP</sub> opening is additionally confirmed, it is intriguing and puzzling that mitoK<sub>ATP</sub> opening could either enhance or attenuate ROS production, depending on the preconditioning phase, ischemia, or reperfusion, and the effect could also be tissue-type specific.

Although DCF is a commonly used probe for ROS, it is not without potential problems. Its reduced acetate form, dichlorodihydrofluorescein (DCFH), can be loaded into mitochondria to monitor locally generated ROS, particularly hydroxyl radical. However, changes of mitochondrial membrane potential or membrane permeability could alter the subcellular dye distribution independent of ROS, thus causing misinterpretation of the results when the compartmentation of the dye cannot be determined, as in the study by Forbes et al. This is particularly problematic when changes in the leakage rate of the dye are undetermined and can even contribute to a decline in total signal in some cases. These problems can be minimized by using less-permeant (e.g., carboxy or chloromethyl) derivatives of DCF. DCF can also be auto-oxidized in the presence of light and may sensitize cells to light-induced radical generation. An important control missing in the study by Forbes et al. is a DCF recording in which only the dye is present. The beginning and endpoints of the time course are taken with no illumination in between. This would definitively rule out DCF-mediated effects on cellular or mitochondrial ROS production.

The role of mitoK<sub>ATP</sub> as a trigger of preconditioning has been demonstrated in isolated as well as in vivo hearts. However, the role of ROS in this trigger effect has only been shown in isolated rabbit<sup>4</sup> and rat<sup>7</sup> hearts. ROS scavengers blocked the protection from diazoxide in both studies, thus strongly implicating a role of ROS in isolated heart model. Again, isolated hearts were perfused with O<sub>2</sub>-saturated buffer with a P<sub>O2</sub> of >500 mm Hg, which may prime the organ for...
ROS production. It will be interesting to see if the ROS involvement can be confirmed in an in vivo setting. Obata et al.\textsuperscript{23} reported that in in vivo rat hearts, cromakalim and nicorandil both increased ROS, measured with a microdilution probe perfused with sodium salicylate, which reacts with hydroxyl radicals produced in the muscle to yield a colored product 2,3-dihydroxybenzoic acid. Glybenclamide and 5-HD both reduced ROS production by K\textsubscript{ATP} openers during ischemia and reperfusion. However, K\textsubscript{ATP} modulators were infused through microdialysis probes along with the ROS probe, making it hard to assess the local concentrations of the compounds throughout the tissue. Furthermore, the observation that K\textsubscript{ATP} inhibitors attenuated ROS production from ischemia and reperfusion is contradictory to other studies.\textsuperscript{19,20}

Because ROS scavengers blocked diazoxide-induced protection, it is a logical explanation that mitoK\textsubscript{ATP} opening in cardiac ventricular myocytes increases ROS production.\textsuperscript{3,7} However, two other possible explanations also exist. First, a mild oxidant environment may be required for the mitoK\textsubscript{ATP} to open or be effective; ie, ROS may have a permissive role, because ROS are known to modulate surface K\textsubscript{ATP},\textsuperscript{24} and a complete elimination of ROS by scavengers could prevent channel opening and thus indirectly abrogate protection. Second, it is also possible that opening of K\textsubscript{ATP} in the cardiac nerve terminal facilitates the release of catecholamines, which then increase ROS production to trigger the protection. Consistent with this idea, pinacidil, a K\textsubscript{ATP} opener, has been shown to increase catecholamine release in isolated guinea pig hearts.\textsuperscript{25} Studies in isolated single myocytes without the presence of neurons may help resolve this issue.

Emerging evidence supports a new paradigm for the roles of mitoK\textsubscript{ATP} in cardioprotection. MitoK\textsubscript{ATP} plays a dual role both as a trigger and mediator of cardioprotection. Protein kinase activation is important during the trigger phase. The link from mitoK\textsubscript{ATP} opening and kinase activation may be ROS, as suggested by two studies.\textsuperscript{3,7} Another link was suggested through an elevation of intracellular Ca\textsuperscript{2+}. The mitoK\textsubscript{ATP} also acts as a mediator; the opening of mitoK\textsubscript{ATP} during the index ischemia is required for protection.\textsuperscript{22} Although the true mechanism of mitoK\textsubscript{ATP} opening as a mediator of protection has not been elucidated, several mechanisms have been proposed, including improvement of cellular energetics, prevention of mitochondrial Ca\textsuperscript{2+} overloading, and an alteration of ROS production during ischemia and reperfusion.\textsuperscript{26} In the future, a full range of studies from the intact animal to the molecular level is needed to uncover and verify the mechanisms.

References

Key Words: preconditioning • diazoxide • K\textsubscript{ATP} channel • mitochondria • reactive oxygen species
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