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Cytoprotective Channels in Mitochondria

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Several ion channels are expressed in the inner and outer membranes of mitochondria, but the exact function of these channels is not completely understood. The opening of certain channels is thought to induce the process of cell death or apoptosis. However, other channels of the inner mitochondrial membrane help protect against ischemic injury and oxidative stress. Mitochondrial ATP-sensitive K^+ channels (mitoK_{ATP}) and mitochondrial Ca^{2+}-activated K^+ channels (mitoK_{Ca}) are the primary protective channels that have been identified. In addition to their thermogenic role, certain isoforms of uncoupling proteins are also shown to have protective roles in certain experimental models. This review attempts to provide an updated overview of the proposed mechanism for the protective function of these membrane proteins. Controversies and unanswered questions regarding these channels will also be discussed.

KEY WORDS: Mitochondria; ischemic preconditioning; mitochondrial ATP-sensitive potassium channel; uncoupling proteins; Ca^{2+}-activated K^+ channel; apoptosis; ion channels.

INTRODUCTION

There are two main pathways of cellular damage and death: necrosis and apoptosis (Nieminen, 2003). Necrosis refers to cell death due to ischemia, injury, radiation, or chemicals. This energy-independent process is irreversible and ultimately culminates in plasma membrane lysis and breakdown of chromosomal DNA into randomly sized fragments. Necrosis induces an inflammatory response that leads to the clearance of cell debris by immigrant phagocytes. This is in contrast to apoptosis, which refers to the process of programmed cell death. Apoptosis occurs in both physiological and pathologic settings and is an energy-dependent process. Unlike necrosis, in apoptosis DNA is broken down into specific sized fragments. Furthermore, metabolism is orderly shut-down followed by minimal leakage of cell contents. Apoptosis does not commonly induce a significant inflammatory response (Green, 2000). Mitochondrial damage is implicated in both necrosis and apoptosis (Desagher and Martinou, 2000; Nieminen, 2003).

The mitochondrial inner membrane was originally thought to have a low permeability to ions (Mitchell, 1961). This was based on the assumption that ion flux would lead to energy dissipation and depolarization of the mitochondrial membrane potential ($\Delta\Psi_m$). However, there is now clear evidence that anion, and monovalent and divalent cation channels exist in the inner membrane of the mitochondria (O'Rourke, 2000b). These channels have profound effects on mitochondrial metabolism and the rate of oxidative phosphorylation. Furthermore, it is now known that the opening of these channels can have either detrimental or beneficial effects on the cell. Table I summarizes the major identified channels in the mitochondrial inner membrane.

An example of a channel with detrimental effects is the mitochondrial permeability transition pore (mPTP). mPTP is a large, Ca^{2+}-sensitive, nonselective channel that can transport molecules as large as 1500 Da (Bernardi *et al*., 2001; Crompton *et al*., 1999; Kroemer *et al*., 1998). It plays a central role in the induction of apoptosis by releasing cytochrome *c* from the intermembrane space (Crompton *et al*., 1999; Heiskanen *et al*., 1999; Liu *et al*., 1996). However, there are also ion channels that protect cells against ischemia and apoptosis.

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Table I. Ion Channels in the Mitochondrial Inner Membrane

Type	Conductance	Primary role	Role in protection or death
Ca^{2+} uniporter	2.6–5.2 pS	Ca^{2+} uptake	Unknown
mPTP	0.03–1.5 nS	Uptake of molecules up to 1500 Da in size	Necrosis, apoptosis
KCa	295–307 pS	Ca^{2+}-activated K^+ uptake	Protection against ischemic damage
KATP	9–100 pS	K^+ uptake	Protection against ischemic damage and apoptosis
UCPs	75 pS	UCP1: thermogenesis	UCP2: Protection against ischemia and oxidative stress
IMAC	15 and 107 pS	Volume regulation	Induction of apoptosis possibly through mPTP

Herein, a review of recent findings on two cytoprotective mitochondrial channels, the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) and the Ca^{2+}-activated K^+ channel (mitoK_{Ca}) is presented. The functional role of mitochondrial uncoupling proteins in cellular protection will also be discussed.

MITOK$_{ATP}$

Evidence for Existence of MitoK$_{ATP}$

The mitochondrial K_{ATP} channel was first identified in 1991 (Inoue *et al*., 1991). Mitoplasts from rat liver mitochondria were used to show a highly selective, small conductance K^+ channel in the inner membrane of the mitochondria. The channel was inhibited by ATP, glybenclamide, and 4-aminopyridine. Using the inside-out patch technique, the conductance of the channel was estimated to be around 9.7 ± 1.0 pS with the pipette and bathing solutions containing 100 and 33.3 mM K^+, respectively. One year after the first report on mitoK_{ATP}, Paucek *et al*. purified a fraction containing mitoK_{ATP} channel activity from the inner membrane of rat liver and beef heart mitochondria (Garlid *et al*., 1996). Using reconstitution studies, they showed that mitoK_{ATP} is competitively inhibited by ATP, ADP, and palmitoyl-coA, and relieved from inhibition by GTP or GDP.

These pioneering works were followed by several reports from multiple labs supporting the existence of this channel. These studies are summarized in Table II and will be briefly discussed here. The reader is also referred to recent reviews on this subject (Ardehali and O'Rourke, 2005; O'Rourke, 2000a; O'Rourke, 2004).

Several studies have suggested a role for the mitoK_{ATP} channel in ischemic preconditioning (IPC), a process in which brief ischemic episodes protect against damage from subsequent longer periods of ischemia (Cohen *et al*., 2000; Kloner *et al*., 1998; Kloner and Yellon, 1994; Murry *et al*., 1986). Diazoxide is >2000 times more potent in opening the mitoK_{ATP} channel than the surface K_{ATP} channels, and this effect is blocked by 5-hydroxydecanoate or 5-HD (Garlid *et al*., 1997). Diazoxide significantly prolongs the time to ischemic contracture, suggesting a protective role against ischemia for mitochondrial rather than surface K_{ATP} channels. This association between mitoK_{ATP} activation and cardioprotection in IPC was supported by studies in isolated rabbit ventricular cells (Liu *et al*., 1998). By measuring the native autofluorescence of mitochondrial flavoproteins and simultaneous measurement of surface K_{ATP} channel current, they found that diazoxide increases flavoprotein oxidation, suggesting that it mainly targets mitoK_{ATP} channels in intact cardiomyocytes. Furthermore, diazoxide decreased cell killing in response to simulated ischemia to about half of that in controls, and 5-HD blocked this protection. Recently, HMR1098, a novel sulfonylurea, was shown to inhibit only surface K_{ATP} channels without suppressing mitoK_{ATP} channels. As expected, HMR1098 does not prevent the cardioprotective effects of IPC and diazoxide (Dhein *et al*., 2000; Fryer *et al*., 2000; Ghosh *et al*., 2000; Jung *et al*., 2000). These studies further support the idea that mitoK_{ATP} rather than surface K_{ATP} channels is involved in cardioprotection. To study the time frame for opening of mitoK_{ATP} channels necessary for protection, Fryer *et al*. applied 5-HD either before or after diazoxide exposure (Fryer *et al*., 2000). They showed that both of these treatments eliminated cardioprotection by diazoxide, suggesting that opening of mitoK_{ATP} channels may cause cardioprotection by both preconditioning and during the long ischemia.

In addition to its role in IPC, mitoK_{ATP} has also been shown to play a role in protecting cardiomyocytes against apoptosis. Pharmacological opening of mitoK_{ATP} channels by diazoxide suppresses several markers of hydrogen peroxide-induced apoptosis, including TUNEL positivity, cytochrome *c* release, caspase-3 activation, and poly (ADP-ribose) polymerase cleavage (Akao *et al*., 2001). In summary, the mitoK_{ATP} channel has been demonstrated to play a key role in IPC and prevention of apoptosis.

Table II. Studies Supporting the Existence of mitoK$_{ATP}$

Approach	Results[a]	References
Patch clamp of intact mitochondria	A single K^+ selective channel Inhibited by ATP, glybenclamide Conductance = 10 pS	Inoue *et al*., 1991
Flavoprotein oxidation	MitoK$_{ATP}$ opening causes mild uncoupling Diazoxide causes net oxidation of flavoproteins reduced to 40% of completely uncoupled state	Liu *et al*., 1998; Sasaki *et al*., 2003; Sasaki *et al*., 2000; Sato *et al*., 2000; Seharaseyon *et al*., 2000
Measurement of mitochondrial volume using light scattering technique	Diazoxide activates mitoK$_{ATP}$ 5-HD, glybenclamide and ATP inhibit the channel	Garlid and Beavis, 1986; Jaburek *et al*., 1998; Szewczyk *et al*., 1993
Effects of mitoK$_{ATP}$ on respiration and membrane potential	Diazoxide stimulates oxygen consumption, a small decrease in $\Delta\Psi_m$ and reduction of cellular ATP levels These effects reversed by 5-HD	Bajgar *et al*., 2001; Debska *et al*., 2002; Kowaltowski *et al*., 2001; Minners *et al*., 2000
Proteoliposome	Diazoxide and pinacidil activate the channel 5-HD, glybenclamide, ADP, and ATP+Mg^{2+} inhibit the channel Channel highly selective for K^+ over Na^+	Bajgar *et al*., 2001; Garlid *et al*., 1997; Paucek *et al*., 1992, 1996; Yarov-Yarovoy *et al*., 1997
Lipid bilayer	A 55-kDa protein has K^+ channel activity with conductance of 30.56 pS	Diwan *et al*., 1988; Grigoriev *et al*., 1999; Mironova *et al*., 1981, 1999, 2001; Paucek *et al*., 1992; Yarov-Yarovoy *et al*., 1997

[a]This table summarizes some of the results in the mitoK$_{ATP}$ field. For a more complete review, please refer to (Ardehali and O'Rourke, 2005; O'Rourke, 2000a, 2004).

Pharmacology of MitoK$_{ATP}$

Several pharmacological reagents have been shown to target mitoK$_{ATP}$. These drugs have been recently reviewed and the reader is referred to these reviews for more detail (Ardehali and O'Rourke, 2005; O'Rourke, 2004).

Mechanism of Protection by MitoK$_{ATP}$

It remains unclear how the opening of mitoK$_{ATP}$ leads to protection against cell death. Three different mechanisms have been proposed (Ardehali and O'Rourke, 2005). These include changes in the levels of reactive oxygen species (ROS), mitochondrial matrix swelling, and changes in the mitochondrial Ca^{2+} levels.

ROS is believed to play a protective role during the preconditioning period and cause injury when it is produced during the reperfusion phase (Forbes *et al*., 2001; Pain *et al*., 2000; Vanden Hoek *et al*., 1998). The opening of mitoK$_{ATP}$ increases ROS production, which is thought to lead to protection. Furthermore, the damaging ROS produced during reperfusion is suppressed by mitoK$_{ATP}$ opening (Ozcan *et al*., 2002; Vanden Hoek *et al*., 2000; Zweier *et al*., 1987). Thus, the opening of mitoK$_{ATP}$ is believed to increase the protective ROS during preconditioning and reduce the harmful ROS during reperfusion.

The opening of mitoK$_{ATP}$ has also been demonstrated by several studies to result in increased mitochondrial matrix swelling (Jaburek *et al*., 1998). Garlid's group has proposed that matrix swelling induced by mitoK$_{ATP}$ opening brings the inner and outer mitochondrial membranes closer to each other, which in turn may promote contact between proteins on these membranes (Kowaltowski *et al*., 2001). The outcome of this effect may be an increase in ADP transport and ATP synthesis. Halestrap's group has proposed an alternative model for protection induced by mitochondrial swelling (Lim *et al*., 2002). They studied isolated mitochondria from Langendorff-perfused hearts after two 5-min cycles of preconditioning followed by longer ischemia. In order to measure mitochondrial matrix volume, ^3H_2O and [^{14}C]sucrose were used. Thirty minutes of ischemia and 30 min of reperfusion resulted in an increase in matrix volume. State 3 respiration and succinate oxidation increased in parallel with matrix volume. However, the addition of diazoxide, a mitoK$_{ATP}$ opener, led to an increase in matrix volume, but a decrease in state-3 respiration. Surprisingly, the mitoK$_{ATP}$ inhibitor 5-HD also increased mitochondrial matrix volume, but inhibited respiration. The authors suggested that since diazoxide inhibited respiration, matrix swelling activation of oxidative phosphorylation is unlikely to be the mechanism by which mitoK$_{ATP}$ induces protection.

Another possible mechanism for protection by mitoK$_{ATP}$ is attenuation of Ca^{2+} uptake by the mitochondria. This has been supported by studies from several groups (Holmuhamedov *et al*., 1999; Liu *et al*., 1998; Murata *et al*., 2001; Wang *et al*., 2001). A recent report showed that diazoxide causes a decrease in mitochondrial Ca^{2+} loading and protection in isolated mitochondria under anoxic conditions (Korge *et al*., 2002). This protection blocked a mitochondrial permeability transition with reoxygenation, which could be the mechanism by which mitoK$_{ATP}$ exerts its anti-apoptotic effects, i.e., blocking mitochondrial permeability transition.

Molecular Structure of MitoK$_{ATP}$

Despite the major role mitoK$_{ATP}$ plays in protection against ischemic cell death, the molecular structure of mitoK$_{ATP}$ remains unclear. The surface K$_{ATP}$ channels are known to be hetero-octamers of four core inward rectifier K^+ channel (Kir6.x) subunits surrounded by four sulfonylurea receptor (SUR) subunits, the latter being members of the ATP-binding cassette (ABC) family (Babenko *et al*., 1998; Seino and Miki, 2003). Garlid has isolated a mitochondrial fraction, which displayed mitoK$_{ATP}$ activity when it was incorporated into artificial lipososmes (Bajgar *et al*., 2001; Paucek *et al*., 1992). This fraction contains a 55- and a 63-kDa protein, which he proposed to be the sulfonylurea-binding protein and pore-forming subunits of mitoK$_{ATP}$, respectively. However, the molecular identity of these proteins has not been identified yet.

Recently, our group took a different approach to study the molecular structure of mitoK$_{ATP}$ (Ardehali *et al*., 2004). Diazoxide has been proposed to inhibit complex II of the respiratory pathway, also known as succinate dehydrogenase or SDH (Schafer *et al*., 1969). Furthermore, SDH inhibitors have cardioprotective effects and attenuate oxidant stress on the heart (Horiguchi *et al*., 2003; Ockaili *et al*., 2001; Ozcan *et al*., 2002). These observations led us to hypothesize that SDH and mitoK$_{ATP}$ physically and functionally interact with each other. Using co-immunoprecipitation, we showed that at least four mitochondrial proteins interact with SDH, possibly as part of a multiprotein complex. These proteins are adenine nucleotide tranlocator, mitochondrial ABC1, ATP synthase, and inorganic phosphate carrier. A mitochondrial fraction containing these four-proteins confered mitoK$_{ATP}$ activity when incorporated into proteoliposomes and lipid bilayers. This channel activity was sensitive to mitoK$_{ATP}$ modulators. Furthermore, addition of SDH inhibitors resulted in a significant decrease in K^+ transport, suggesting that SDH may influence mitoK$_{ATP}$ through its physical and functional interaction with this multiprotein complex.

Despite recent advances in our understanding of mitoK$_{ATP}$, several questions remain regarding this channel that need to be answered. The selectivity of mitoK$_{ATP}$ modulators is a controversial issue and needs further investigation. The molecular structure of mitoK$_{ATP}$ is also unclear, although the recent findings suggest a macromolecular complex may constitute the structure of this channel. However, the identity of the pore-forming unit of the channel is not understood. Finally, several studies have questioned the selectivity of this channel for K^+.

MITOK$_{CA}$

K_{Ca} channels were first identified in red blood cells, where their opening resulted in cell shrinkage and membrane hyperpolarization (Gardos, 1958). In the nervous system, K_{Ca} currents have been shown to underlie the afterhyperpolarization that follows bursts of action potentials in the mammalian hippocampus (Alger and Nicoll, 1980; Hotson and Prince, 1980; Schwartzkroin and Stafstrom, 1980). K_{Ca} currents can be activated by Ca^{2+} and lack voltage sensitivity. They also have different pharmacological profiles, i.e., some are blocked by the bee-venom toxin apamin, whereas others are not.

Siemen was the first to report the presence of K_{Ca} channels in the mitochondria (Siemen *et al*., 1999). Using the patch clamp technique in mitoplasts of the human glioma cell line LN229, they recorded a K^+ selective channel with a conductance of 295 ± 18 pS in the presence of 150 mM KCl. The open probability of the channel increased with increasing Ca^{2+} concentrations, and was reduced by the addition of charybdotoxin.

The characterization of a mitochondrial K_{Ca} led O'Rourke's group to hypothesize that this channel may have a cytoprotective role (Xu *et al*., 2002). They first showed that a Ca^{2+}-activated K^+ channel is present in the mitochondria of cardiomyocytes. Using mitoplasts from cardiac myocytes, single channel currents with a conductance of 307 ± 4.6 pS were recorded in the presence of 150 mM K^+ and 512 nM of Ca^{2+}. The channel was activated by raising Ca^{2+} in the media and was inhibited by 200 nM of charybdotoxin. They then showed that K_{Ca} contributes significantly to the total K^+ transport into the mitochondria. A K^+ fluorescent marker was trapped inside intact mitochondria, followed by increasing the bath K^+ levels from 0 to 5 mM over <2 s. This method displayed an increase in the K^+ flux into the matrix in response to raising K^+ in the bath solution. The effect was activated by the K^+ ionophore valinomycin and was inhibited by charybdotoxin. The presence of an $\sim$80 kDa protein that

binds to a BK subtype of K_{Ca} was confirmed in the mitochondrial membrane using immunoblot analysis of one and two dimensional gels.

To evaluate the cytoprotective role of this channel, perfused hearts were subjected to ischemia and reperfusion after addition of a K_{Ca} opener, NS-1619 in the presence and absence of the K_{Ca} inhibitor paxilline. NS-1619 increased coronary blood flow by 66% at 30 μM, which was attributed to the binding of the chemical to the vascular isoform of the K_{Ca} channel. Under ischemic conditions, pretreatment with NS-1619 attenuated the ischemic damage to an extent similar to that exerted by the mitoK_{ATP} opener diazoxide. Paxilline alone did not influence the infarct size, but blocked the protective effects of NS-1619.

These results demonstrated the existence of a K_{Ca} channel in the mitochondrial inner membrane. The channel contributes to the overall K^+ influx into the mitochondrial matrix. Furthermore, the opening of this channel has physiological effects, inducing cytoprotection under ischemic conditions. However, these pioneering studies have raised many new questions about mitochondrial channels. The molecular structure of mitoK_{Ca} is not understaood. The individual contribution of mitoK_{ATP} and mitoK_{Ca} channels to total cellular protection is not clear, nor are the downstream effects of the opening of these channels. Furthermore, the inciting factors and intracellular pathways that lead to opening of each of these channels needs to be investigated.

UNCOUPLING PROTEINS

The uncoupling proteins (UCPs) comprise a family of mitochondrial inner membrane proteins, which generate heat by uncoupling ATP synthesis from substrate oxidation and electron transport (Argiles *et al*., 2002). The uncoupling proteins dissipate the proton gradient across the mitochondrial inner membrane by promoting proton leak. Five UCP isoforms have been characterized in mammalian cells: UCP1, UCP2, UCP3, UCP4, and brain mitochondrial carrier protein-1 (BMCP-1). UCP1, also known as thermogenin, is a 32-kDa protein that is expressed exclusively in brown adipose tissue and is a key player in thermogenesis (Nicholls and Locke, 1984; Ricquier and Bouillaud, 2000). An anion channel activity with a conductance of 75 pS was detected with UCP1 (Huang and Klingenberg, 1996), suggesting that this protein may also have ion channel activity under certain conditions. While UCP2 is expressed ubiquitously, UCP3 is expressed predominantly in skeletal muscles (Pecqueur *et al*., 2001). UCP3 was recently shown not to function as a mitochondrial uncoupler in human muscle in subjects fed a high-fat diet (Hesselink *et al*., 2003). BMPC-1 and UCP4 are mainly expressed in neuronal tissues and are capable of uncoupling respiration, at least when expressed in yeast (Mao *et al*., 1999; Sanchis *et al*., 1998).

UCP2 controls ROS production and contributes to the immune response during infection (Arsenijevic *et al*., 2000). Furthermore, in pancreatic β-cells, UCP2 inhibits glucose-stimulated insulin secretion by regulating ATP production (Chan *et al*., 2001; Joseph *et al*., 2002; Zhang *et al*., 2001). A recent report by Mattiasson *et al*. provided evidence that UCP2 may be neuroprotective by attenuating mitochondrial accumulation of ROS and reducing mitochondrial Ca^{2+} uptake (Mattiasson *et al*., 2003). The authors first showed that UCP2 levels are increased when rat brain was exposed to a 3 min period of ischemia followed by 10 min of ischemia 2 days later (IPC model in the brain). Overexpressing UCP2 in mice attenuated trauma- and ischemia-induced brain damage, and led to enhanced neurological recovery. In primary culture cells, UCP2 overexpression also protected neurons that were subjected to oxygen/glucose deprivation. Similar neuroprotective effects were noted with the uncoupling agent, dinitrophenol. Thus, Mattiasson *et al*. concluded that the neuroprotective effects of UCP2 are likely due to its mitochondrial uncoupling properties. They suggested that UCP2 may direct ROS from the mitochondrial matrix to the cytosol as a possible mechanism of protection.

The protective effects of UCP2 were recently confirmed in cardiomyocytes. Teshima *et al*. (2003) overexpressed human UCP2 using an adenoviral vector in cultured neonatal rat cardiomyocytes. UCP2 overexpression attenuated several markers of hydrogen peroxide-induced apoptosis, including TUNEL positivity, caspase-3 activation, and propidium iodide uptake. It also significantly suppressed oxidant-induced loss of mitochondrial membrane potential. Ca^{2+} overload and mitochondrial ROS production, both contributors to the mitochondrial membrane potential loss, were significantly suppressed in response to UCP2 overexpression. These results suggest that UCP2 may have protective effects against ischemia and oxidant stress, however, the mechanism by which this protein exerts its protective effect is not completely understood.

CONCLUSIONS

In the past couple of decades, different techniques such as patch clamp recordings, mitochondrial swelling assays, and reconstitution of mitochondrial proteins in lipid bilayers or proteolipososmes have been employed

to show that a portion of the mitochondrial ion leakage is mediated by currents with ion selectivity and specific conductances. Although these currents have a significant effect on mitochondrial metabolism and function, there is very little known about their molecular structures, and their role in physiological and pathological conditions. There is now abundant evidence that some of these currents are capable of exerting protective effects under pathological conditions, such as ischemia and oxidant stress. MitoK_{ATP} and mitoK_{Ca} are prime examples of ion channels in the mitochondria with protective roles. The opening of mitoK_{ATP} is believed to be the key intermediate step in IPC and prevention of apoptosis. The underlying mechanism is not totally understood, however, changes in mitochondrial Ca^{2+} uptake, ROS production and matrix swelling have been proposed as likely mechanisms. Despite the important role mitoK_{ATP} plays in IPC, the molecular structure of this protein is not clear. We recently showed that a multiprotein complex containing SDH confers mitoK_{ATP} activity, however, the pore-forming unit of the channel is not characterized yet. mitoK_{Ca} was recently identified as a cytoprotective channel and was shown to contribute to the overall K^+ transport into the mitochondria. There are many questions that remain to be answered regarding this channel and its overall contribution to cellular protection.

UCPs are traditionally known to uncouple ATP synthesis from oxidative phosphorylation by transporting protons across the mitochondrial inner membrane. However, it is now known that UCP2 also plays a protective role against ischemia and oxidative stress. The mechanism for this effect is not well understood, however, shifting ROS from the mitochondria to the cytoplasm is proposed as a possible mechanism. The uncoupling function of UCP2 remains a controversial issue as well as its tissue localization. Furthermore, the cardioprotective effects of this protein need to be studied in adult hearts of intact animals and in other tissues where it is expressed.

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