Mitochondrial ATP-Sensitive K$^+$ Channels Prevent Oxidative Stress, Permeability Transition and Cell Death

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Ischemia followed by reperfusion results in impairment of cellular and mitochondrial functionality due to opening of mitochondrial permeability transition pores. On the other hand, activation of mitochondrial ATP-sensitive K$^+$ channels (mitoK$_{ATP}$) protects the heart against ischemic damage. This study examined the effects of mitoK$_{ATP}$ and mitochondrial permeability transition on isolated rat heart mitochondria and cardiac cells submitted to simulated ischemia and reperfusion (cyanide/aglycemia). Both mitoK$_{ATP}$ opening, using diazoxide, and the prevention of mitochondrial permeability transition, using cyclosporin A, protected against cellular damage, without additive effects. MitoK$_{ATP}$ opening in isolated rat heart mitochondria slightly decreased Ca^{2+} uptake and prevented mitochondrial reactive oxygen species production, most notably in the presence of added Ca^{2+}. In ischemic cells, diazoxide decreased ROS generation during cyanide/aglycemia while cyclosporin A prevented oxidative stress only during simulated reperfusion. Collectively, these studies indicate that opening mitoK$_{ATP}$ prevents cellular death under conditions of ischemia/reperfusion by decreasing mitochondrial reactive oxygen species release secondary to Ca^{2+} uptake, inhibiting mitochondrial permeability transition.

KEY WORDS: Free radicals; preconditioning; potassium; calcium; anoxia/reoxygenation.

INTRODUCTION

Prolonged ischemia followed by reperfusion leads to cardiac tissue damage and is a leading cause of death and disability worldwide (Tunstall-Pedoe *et al.*, 1999). Ischemic heart injury can be reduced by short periods of non-lethal ischemia/reperfusion before the damaging ischemic event, a phenomenon known as ischemic preconditioning (Murry *et al.*, 1986). This powerful protective mechanism is effective both in whole hearts and cultured cell models of ischemia/reperfusion (Takasaki *et al.*, 1999; Vanden Hoek *et al.*, 2000; Marber, 2000; Laclau *et al.*, 2001; Seymour *et al.*, 2002).

The understanding of the processes through which ischemic preconditioning exerts its protective effects has been a central focus of recent literature. One mechanism that is directly related to the protection triggered by preconditioning is the opening of ATP-sensitive K$^+$ channels (mitoK$_{ATP}$) in the mitochondrial inner membrane. Indeed, mitoK$_{ATP}$ agonists can induce protective effects similar to preconditioning (Garlid *et al.*, 1997; Vanden Hoek *et al.*, 2000) while mitoK$_{ATP}$ antagonists (Jaburek *et al.*, 1998) prevent the beneficial effects of ischemic preconditioning (Gross and Auchampach, 1992; Auchampach *et al.*, 1992; Vanden Hoek *et al.*, 2000).

MitoK$_{ATP}$ opening appears to promote enhanced post-ischemic survival by affecting multiple mitochondrial and cellular functions (see Liu *et al.*, 1999; Garlid *et al.*, 2003; O'Rourke, 2004 for reviews). Under physiological conditions, mitoK$_{ATP}$ openers have few direct effects on mitochondrial respiration, membrane potential, or Ca^{2+} uptake. Matrix volume is enhanced by mitoK$_{ATP}$

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Key to abbreviations: 5-hydroxydecanoate, 5-HD; arbitrary units, A.U.; cyclosporin A, CsA; diazoxide, DZX; 2′,7′-dichlorodihydro-fluorescein, H$_2$DCF; 2′,7′-dichlorodihydrofluorescein diacetate, H$_2$DCF-DA; 2′,7′-dichlorofluorescein, DCF; mitochondrial ATP-sensitive K$^+$ channels, mitoK$_{ATP}$; mitochondrial permeability transition, MPT; reactive oxygen species, ROS.

activity under physiological conditions and when respiration is inhibited, as occurs during ischemia (Kowaltowski *et al*., 2001a). Increases in mitochondrial volume may lead to improved post-ischemic oxidative phosphorylation due to optimized transport of substrates across mitochondrial membranes (dos Santos *et al*., 2002) and may also contribute toward energetic preservation of the tissue during ischemia, by preventing ATP hydrolysis due to the reverse activity of the ATP synthase (dos Santos *et al*., 2002; Belisle and Kowaltowski, 2002).

Other studies have suggested that an important effect of mitoK$_{ATP}$ opening is the prevention of enhanced mitochondrial Ca^{2+} uptake during ischemia (Murata *et al*., 2001; Ishida *et al*., 2001; Belisle and Kowaltowski, 2002). Although the changes in membrane potential under respiring conditions are not sufficient to reduce the driving force for Ca^{2+} uptake (Kowaltowski *et al*., 2001b), proton pumping during ischemia is maintained by ATP hydrolysis due to the reverse activity of the ATP synthase (Rego *et al*., 2001). MitoK$_{ATP}$ opening decreases the formation of an inner membrane potential under these conditions by inhibiting ATP transport into mitochondria (dos Santos *et al*., 2002; Belisle and Kowaltowski, 2002). Lower Ca^{2+} accumulation during ischemia and reperfusion may prevent the occurrence of the mitochondrial permeability transition (MPT) (Murata *et al*., 2001; Halestrap *et al*., 1998), a non-selective permeabilization of the inner membrane which results in loss of organelle function and may promote cell death (Zoratti and Szabo, 1995; Kowaltowski and Vercesi, 1999; Kowaltowski *et al*., 2001). Indeed, MPT has been widely shown to be a decisive event in post-ischemic cardiac damage (Halestrap *et al*., 1997, 1998; Hausenloy *et al*., 2003, 2004).

MPT is caused by mitochondrial Ca^{2+} overload, oxidative stress, adenine nucleotide depletion and elevated phosphate concentrations (Zoratti and Szabo, 1995; Kowaltowski and Vercesi, 1999; Kowaltowski *et al*., 2001), conditions prevailing during ischemia/reperfusion. In addition to preventing Ca^{2+} uptake, decreases in ATP levels and increases in cellular inorganic phosphate during ischemia, we have suggested mitoK$_{ATP}$ opening may also prevent the generation of mitochondrial reactive oxygen species (ROS) (Ferranti *et al*., 2003), although this idea is controversial (Forbes *et al*., 2001; see Gross, 2003; for a critical review). Mitochondria continuously generate a small quantity of ROS, mainly due to monoelectronic reduction of oxygen to superoxide radical anions by electrons sidetracked from early steps in the electron transport chain. Many distinct pathways that mildly uncouple respiration from oxidative phosphorylation strongly prevent mitochondrial ROS release, by decreasing the probability of electron leakage from Complexes I and III (Korshunov

et al., 1997; Starkov, 1997; Miwa and Brand, 2003). Indeed, recent data have suggested that uncoupling itself may be cardioprotective (Rodrigo *et al*., 2002).

In this manuscript, we evaluated the relationship between cardiac protection promoted by mitoK$_{ATP}$ opening and MPT prevention. The roles of cellular and mitochondrial oxidative stress, in addition to mitochondrial Ca^{2+} uptake, were addressed. Our results indicate that mitoK$_{ATP}$ and MPT inhibition act in the same pathway toward the prevention of cardiac cell death and that, under non-respiring conditions, mitoK$_{ATP}$ opening can strongly inhibit oxidative stress and MPT promoted by ischemia/reperfusion.

MATERIALS AND METHODS

Cell Cultures

Cardiac HL-1 cells were developed and kindly donated by William C. Claycomb. These cells maintain their cardiac phenotype during extended passages, present ordered myofribrils, cardiac-specific junctions and several voltage-dependent currents that are characteristic of a cardiac myocyte phenotype (Claycomb *et al*., 1998). Furthermore, HL-1 cells present conserved preconditioning mechanisms dependent on protein kinase C and K$^+$ channel activation (Seymour *et al*., 2003). For routine growth, HL-1 cells were maintained in T-75 flasks at 37°C in an atmosphere of 5% CO$_2$ in Claycomb medium (JRH Biosciences) supplemented with 0.1 mM norepinephrine, 100 U/ml and 100 μg/ml penicillin/streptomycin, 2 mM glutamine and 10% fetal bovine serum. Experiments were conducted at 100% confluence, after trypsinization and resuspension in a standard buffer (pH = 7.4) containing (in mmol/L): NaCl, 137; Hepes, 5; glucose, 22; taurine, 20; creatine, 5; KCl, 5.4; MgCl$_2$ 1; sodium pyruvate, 5 and CaCl$_2$, 1.

Simulated Ischemia/Reperfusion

Ischemia was simulated by metabolic inhibition and substrate deprivation using 500 μM potassium cyanide and 2 mM 2-deoxyglucose, added to standard buffer devoid of glucose and sodium pyruvate. HL-1 myocytes were incubated under CN/aglycemia during 60 min followed by 5 min centrifugation and re-suspension of the cell pellet in control buffer for simulated reperfusion. Diazoxide (30 μM) or 1 μM cyclosporin A were pre-incubated for 20 min before CN/aglycemia. Control HL-1 myocytes were incubated with standard buffer solution

during the entire experimental period, and submitted only to the centrifugations in order to equal mechanical damage.

Cell Viability

Cell viability was assessed by relative fluorescence of 50 μM ethidium bromide (Sigma-Aldrich) using a Hitachi F4500 spectrofluorometer at excitation and emission wavelengths of 365 and 580 nm, respectively (Karsten, 1980). Cells were permeabilized with 0.005% digitonin at the end of the each experiment to promote 100% cell death. The auto-fluorescence of ethidium bromide was subtracted from total fluorescence in the presence of cells, ethidium bromide or digitonin. Data are expressed as the percentage of total cells.

Intracellular ROS Generation

ROS production by HL-1 cells was followed by the conversion of 2′,7′-dichlorodihydrofluorescein (H$_2$DCF) to highly fluorescent 2′,7′-dichlorofluorescein (DCF). Cells were loaded with 10 μmol/L H$_2$DCF-diacetate (H$_2$DCF-DA, Sigma-Aldrich) for 1 h at 37°C, in the dark. After loading, cells were washed to remove extracellular dye. Intracellular DCF fluorescence was accompanied using a Hitachi F4500 spectrofluorometer at excitation and emission wavelengths of 488 and 530 nm, respectively, and slit widths of 5.0 nm, at 37°C. Data are expressed as the percentage of control fluorescence at each time point, to avoid plotting fluorescence differences promoted by washes.

Isolation of Rat Heart Mitochondria

Mitochondria were isolated from male Sprague-Dawley rats as described by Kowaltowski *et al.* (2001). Briefly, rats hearts were removed and immediately washed in ice-cold buffer containing 300 mM sucrose, 10 mM K$^+$ Hepes buffer, pH 7.2 and 1 mM K$^+$ EGTA. The tissue was minced finely and incubated in the presence of protease Type I (Sigma-Aldrich) during 10 min. Excess protease was removed by washing the heart fragments with the same buffer containing 1 mg/mL BSA. The tissue was then homogenized manually. Nuclei and cellular residues were pelleted by centrifugation at 600g for 5 min, and the supernatant re-centrifuged at 9000g for 8 min. The mitochondrial pellet was washed twice to eliminate contaminating blood and re-suspended in a minimal amount of buffer ($\sim$200 μL). Mitochondria were kept over ice until the experiments were initiated, at 37°C. To ensure mitoK$_{ATP}$ activity and pharmacological regulation, all experiments using isolated mitochondria were conducted within 1 h of isolation.

Mitochondrial ROS Generation

H$_2$DCF-DA (10 μmol/L) was used to monitor ROS release rates in rat heart mitochondrial suspensions. ROS production was measured using a temperature-controlled Hitachi F4500 spectrofluorometer at excitation and emission wavelengths of 488 and 530 nm, respectively.

Mitochondrial Ca^{2+} Uptake

Calcium uptake by isolated heart mitochondria was monitored following the fluorescence of 0.1 μM calcium green-5N hexapotassium salt (Molecular Probes) in a Hitachi F4500 spectrofluorometer operating in excitation and emission wavelengths of 506 and 531 nm, respectively, and slit widths of 5.0 nm (Murphy *et al.*, 1996). Fluorescence was continuously monitored at 37°C and pulses of CaCl$_2$ were added until there was no evidence of further uptake of the cation. Total Ca^{2+} uptake was calculated adding all pulses in each trace.

Statistical Analysis

Data were analyzed using Sigmastat®. The difference between experimental groups was analyzed using one-way analysis of variance (ANOVA) with multiple pairwise Tukey tests for post hoc analysis or Student's *t*-tests. Data shown as traces are representative experiments of at least three similar repetitions. Symbol and bar graphs represent averages $\pm$ SE. $p < 0.05$ was considered significant.

RESULTS

Although both mitoK$_{ATP}$ opening and MPT prevention are known to be cardioprotective during ischemia (Murata *et al.*, 2001; Vanden Hoek *et al.*, 2000; Garlid *et al.*, 1997; Halestrap *et al.*, 1997, 1998; Hausenloy *et al.*, 2003, 2004), it is unclear if the effects of these interventions are additive or complementary. Figure 1 illustrates the effect of mitoK$_{ATP}$ opening and MPT inhibition on cardiac HL-1 cellular viability during simulated ischemia
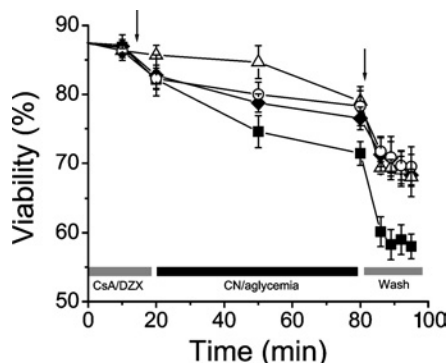
Fig. 1. Cardiac cell death promoted by CN/aglycemia is prevented by activation of mitoK$_{ATP}$ and MPT inhibition. HL-1 cells were pre-incubated in standard media for 20 min in the presence of 30 μM DZX (♦) or 1 μM CsA (△). CN/aglycemia cells (♦, △ and ■) were incubated in media containing CN$^-$ and deoxyglucose during the time period indicated, as described in Materials and Methods. All samples, including controls (O), were centrifuged and re-suspended in fresh media where indicated by the arrows (see Materials and Methods). Data are show as means ± SE of at least four repetitions.

(CN/aglycemia) followed by reestablishment of oxidative phosphorylation (Murata *et al.*, 2001). A variety of protocols with different CN$^-$ concentrations and incubation times was tested until a condition with a mild loss of cellular viability was found. Under this condition, pretreatment with the mitoK$_{ATP}$ agonist diazoxide (DZX, 30 μM, ♦) had a maximal protective effect ($p = 0.019$ versus CN/aglycemia, ■, at 10 min reperfusion). The protective effect of DZX was completely reversed by 150 μM 5-hydroxydecanoate (5-HD, by 102 ± 24%) or 2 μM glybenclamide (by 94 ± 10%), which are effective mitoK$_{ATP}$ antagonists (results excluded from the figure for clarity). MPT prevention using cyclosporin A (CsA, 1 μM, △) also had a very strong protective effect ($p = 0.014$ versus CN/aglycemia at 10 min reperfusion). Both DZX and CsA lead to final viability levels indistinguishable from control cells (O), which were submitted only to centrifugations. Thus, the effects of DZX and CsA are clearly not additive.

Based on previous literature results indicating that mitoK$_{ATP}$ opening prevents many conditions that trigger MPT such as ATP depletion, Ca^{2+} uptake and oxidative stress (Belisle and Kowaltowski, 2002; Ferranti *et al.*, 2003), we hypothesized that mitoK$_{ATP}$ could prevent conditions that favor the opening of MPT pores under ischemic conditions. In order to verify this possibility, we conducted experiments using isolated heart mitochondria incubated under non-respiratory conditions. Since MPT is caused by excessive Ca^{2+} accumulation associated to mitochondrial oxidative stress (Kowaltowski *et al.*, 2001),

both mitochondrial Ca^{2+} uptake and ROS release were measured.

We have previously shown that Ca^{2+} uptake rates in respiring mitochondria are unaffected by mitoK$_{ATP}$ opening (Kowaltowski *et al.*, 2001). However, results from our group and others (Murata *et al.*, 2001; Ishida *et al.*, 2001; Belisle and Kowaltowski, 2002) suggest that mitoK$_{ATP}$ opening can prevent Ca^{2+} uptake under ischemic conditions. In Figure 2, we directly measured maximal Ca^{2+} uptake capacity under non-respiring conditions. In the presence of added ATP to support the mitochondrial inner membrane potential, Ca^{2+} uptake rates were slightly decreased by DZX (Fig. 2(a)). However, this difference was on average small and total Ca^{2+} uptake, which is determinant for MPT occurrence, was only marginally decreased by DZX (Fig. 2(b)). Thus, it seems improbable that differences in Ca^{2+} uptake, alone, account for the prevention of MPT by mitoK$_{ATP}$ opening in non-respiratory conditions.

We then investigated mitoK$_{ATP}$ effects on ROS release. We have previously shown that mitoK$_{ATP}$ opening can prevent mitochondrial ROS release in a mechanism related to mild uncoupling promoted by the activity of this channel and K$^+$/H$^+$ exchange (Ferranti *et al.*, 2003). The experiments shown in Fig. 3 were conducted to verify if mitoK$_{ATP}$ could also prevent ROS release under non-respiratory conditions, in the presence or absence of Ca^{2+}. H$_2$DCF was used as a probe since it can function in the presence of CN$^-$, even though it is less sensitive than other horseradish peroxidase-based methods. Despite this lower sensitivity, we found that DZX reproducibly decreased H$_2$DCF-measured mitochondrial ROS release in the presence of CN$^-$, in a manner sensitive to the mitoK$_{ATP}$ antagonist 5-HD (Panel A). When Ca^{2+} was added, the preventive effect of DZX was largely enhanced (Panels B and C). This indicates that mitoK$_{ATP}$ opening is an efficient mechanism to prevent ROS release from non-respiring mitochondria, and has an enhanced effect when Ca^{2+} is present.

In order to confirm the possibility that mitoK$_{ATP}$ opening reduces mitochondrial ROS release and, thus, prevents MPT and cell death, we returned to the cardiac cell CN/aglycemia model and measured intracellular H$_2$DCF oxidation in the presence of DZX and CsA (Fig. 4). DCF fluorescence was plotted as levels relative to control (untreated) cells at each time point to compensate for fluorescence changes promoted by washes. We found that CN/aglycemia (■) promoted two bursts in DCF fluorescence, one soon after respiratory and metabolic inhibition and one after the wash that simulates reperfusion. CsA (△) prevented the second increase in DCF fluorescence, which may, therefore, occur in response to
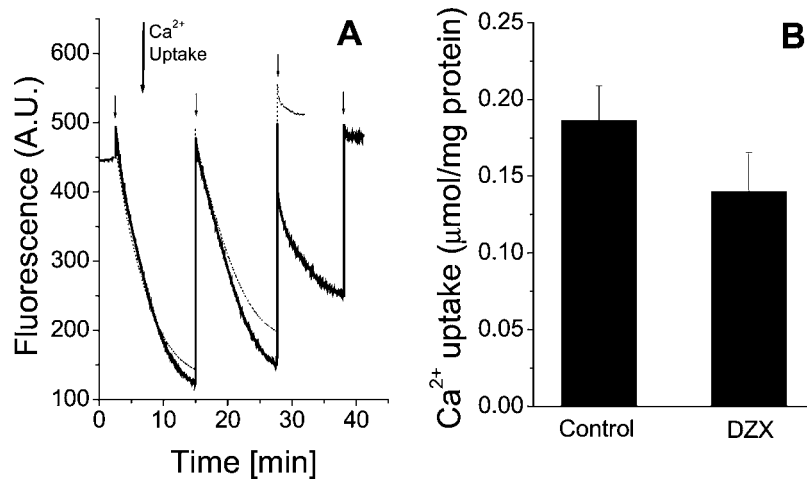
Fig. 2. MitoK$_{ATP}$ opening effects on mitochondrial Ca^{2+} uptake under non-respiratory conditions. Mitochondria ($\sim$0.8 mg/ml) were incubated at 37°C in 10 mM Hepes, 2 mM pyruvate, 2 mM malate, 2 mM MgCl$_2$, 150 mM KCl, 2 mM KH$_2$PO$_4$, 1 mM ATP and 500 μM KCN pH 7.2 (KOH), in the presence of 0.1 μM Ca^{2+} Green to measure extramitochondrial Ca^{2+}. Panel A: Typical Ca^{2+} uptake trace, in the presence (full line) or absence (dotted line) of 40 μM DZX. Ca^{2+} (sufficient to increase free Ca^{2+} levels to 60 μM) was added where indicated by the arrows. Panel B: Average total Ca^{2+} uptake (mean/mg mitochondrial protein, $n = 6$).

MPT itself or be a consequence of cellular degradation. CsA did not have any significant effect on the increase of DCF fluorescence observed soon after CN/aglycemia. Conversely, DZX ($\blacklozenge$) significantly prevented both bursts in H$_2$DCF oxidation. This finding is compatible with the idea that mitoK$_{ATP}$ opening prevents mitochondrial oxidative stress, resulting in decreased MPT and less cell death.

DISCUSSION

Using cardiac cells submitted to CN/aglycemia, we demonstrate here that mitoK$_{ATP}$ opening and MPT inhibition act on the same pathway toward the prevention of cell death, and do not present additive effects (Fig. 1). DCF fluorescence measurements of ROS levels within these cells indicate the participation of mitoK$_{ATP}$ is upstream
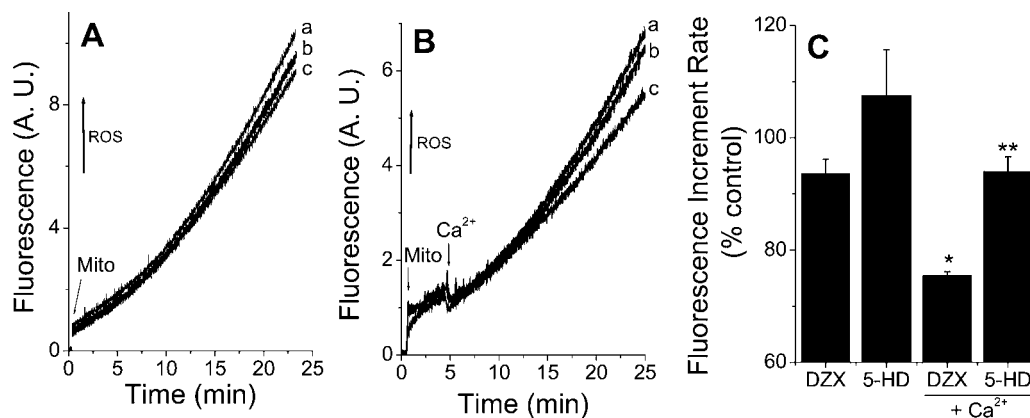

Fig. 3. MitoKATP opening prevents Ca^{2+}-stimulated mitochondrial ROS release. Mitochondria ($\sim$0.6 mg/ml) were incubated under the conditions described in Fig. 2, in the presence of 10 μM H$_2$DCF to measure mitochondrial ROS and no further additions (lines a), 30 μM DZX (lines b) or 30 μM DZX and 150 μM 5-HD (lines c). In Panel B, Ca^{2+} (sufficient to increase free Ca^{2+} levels to 200 μM) was added where indicated. Panel C represents averages of 3–6 experiments such as those in Panels A and B. $^{*}p < 0.05$ relative to DZX; $^{**}p < 0.05$ relative to DZX plus Ca^{2+}.
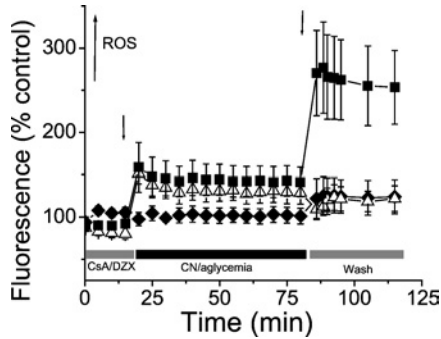
Fig. 4. DZX prevents oxidative stress during and after CN/aglycemia. HL-1 cells were pre-loaded with H_2DCF (see Materials and Methods) and submitted to CN/aglycemia ($\blacklozenge$, $\triangle$ and $\blacksquare$) in the presence of DZX ($\blacklozenge$) or CsA ($\triangle$), under the conditions described in Fig. 1. Data are shown as means of fluorescence relative to fluorescence in H_2DCF-loaded control cells.

of MPT inhibition (Fig. 4), as has been hypothesized previously (Hausenloy *et al.*, 2004). This prevention is associated both with a slight decrease in maximal Ca^{2+} uptake capacity (Fig. 2) and a more substantial prevention of mitochondrial ROS release in the presence of added Ca^{2+} (Fig. 3). Thus, our results suggest that the preventive effects of DZX on Ca^{2+} uptake and ROS release act synergistically, reducing oxidative stress resulting

from excess mitochondrial Ca^{2+} accumulation. The relevance of these findings toward events in the intracellular environment is supported by experiments demonstrating that DZX-induced mitoK$_{ATP}$ opening also decreases ROS in intact cells during and after CN/aglycemia (Fig. 4). Together, the results suggest that mitoK$_{ATP}$ opening improves redox balance by preventing mitochondrial ROS release and, in this manner, inhibits MPT (a consequence of mitochondrial oxidative stress; Kowaltowski *et al.*, 2001) and cell death (see Fig. 5.)

Our findings suggesting the concomitant decrease in Ca^{2+} uptake and ROS release is a key mechanism in mitoK$_{ATP}$-induced cell death prevention are in line with previous results demonstrating that mitoK$_{ATP}$ opening inhibits mitochondrial Ca^{2+} uptake during ischemia (Murata *et al.*, 2001; Ishida *et al.*, 2001; Belisle and Kowaltowski, 2002). They are also compatible with results from our group indicating that DZX prevents ROS release from isolated respiring mitochondria (Ferranti *et al.*, 2003). They are, however, at odds with data from other groups suggesting that mitoK$_{ATP}$ opening within cells enhances ROS release (Forbes *et al.*, 2001; Krenz *et al.*, 2002), and that the protective effects of DZX can be prevented by antioxidants (Forbes *et al.*, 2001; Carroll *et al.*, 2001). We find these results questionable because
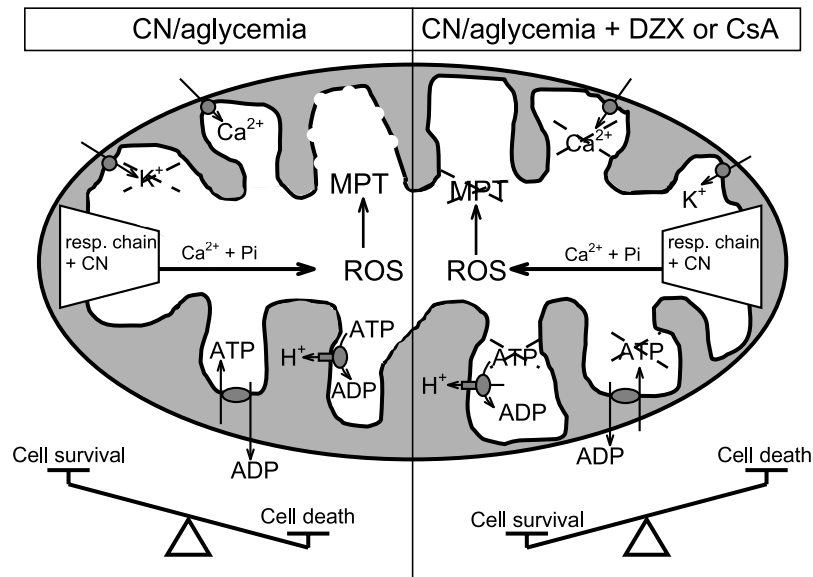

Fig. 5. Consequences of mitoK$_{ATP}$ opening and MPT prevention during CN/aglycemia. Inhibition of the mitochondrial respiratory chain (Resp. Chain) by CN^- leads to depletion of intracellular ATP due to the lack of oxidative phosphorylation and reverse activity of the ATP synthase. Lack of ATP and increased Ca^{2+} and inorganic phosphate (Pi) uptake lead to increased ROS production and, consequently, MPT. MitoK$_{ATP}$ opening by DZX inhibits MPT by decreasing ATP hydrolysis, Ca^{2+} uptake and ROS production. MPT can also be directly inhibited by cyclosporin A, favoring cell survival.

MitoTracker Red, used as an intracellular mitochondrial ROS probe in one of the studies, does not respond to classical uncouplers or respiratory inhibitors (Krenz *et al.*, 2002), indicating it is not an adequate mitochondrial ROS probe under these conditions. Furthermore, we have recently determined, using mitochondrial inhibitors, that the small increment in DCF fluorescence observed upon DZX addition (Forbes *et al.*, 2001) is unrelated to mitochondrial ROS release (Facundo and Kowaltowski, unpublished results, see Gross, 2003; for a critical review). Finally, although the use of antioxidants to reverse the effects of DZX could be a strong indication of a ROS role downstream of mitoK$_{ATP}$ activation, the only class of antioxidants used to date is of thiol reducing agents (Forbes *et al.*, 2001; Carroll *et al.*, 2001), which can reverse pre-existing protein oxidation promoted prior to mitoK$_{ATP}$ opening. Indeed, both our group (Fig. 4) and others (Vanden Hoek *et al.*, 2000; Ozcan *et al.*, 2002) have measured a large decrease in cellular ROS promoted by mitoK$_{ATP}$ agonists during ischemia/reperfusion. This finding, added to evidence that mitoK$_{ATP}$ prevents ROS release in isolated mitochondria (Ferranti *et al.*, 2003), strongly supports a role of these channels in the prevention of mitochondrial ROS release under both physiological and pathological conditions.

In respiring mitochondria, the prevention of ROS release by mitoK$_{ATP}$ is related to the mild uncoupling effect of this channel acting concurrently with K$^+$/H$^+$ exchangers (Korshunov *et al.*, 1997; Kowaltowski *et al.*, 2001; Ferranti *et al.*, 2003). It is thus surprising that mitoK$_{ATP}$ opening can also prevent ROS release in CN$^-$-poisoned mitochondria, in which no uncoupling can occur. This effect is much more pronounced in the presence of Ca^{2+} (Fig. 3), suggesting it involves the synergistic result of mitoK$_{ATP}$ in preventing Ca^{2+} uptake and ROS release. Indeed, Ca^{2+} has been previously shown to lead to mitochondrial oxidative stress (Grijalba *et al.*, 1999; Kowaltowski *et al.*, 1999, 2001), and even the small decrease in Ca^{2+} loading (Fig. 2) observed upon mitoK$_{ATP}$ opening may be sufficient to significantly prevent ROS release. On the other hand, recent evidence indicates that DZX regulates K$^+$ transport by inhibiting succinate dehydrogenase, a proposed component of the mitoK$_{ATP}$ complex (Ardehali *et al.*, 2004). It is possible this regulation also involves providing fewer electrons to complexes within the inhibited respiratory chain in which superoxide radical anions can be formed. The suggestion that mitoK$_{ATP}$ agonists can prevent cell death by decreasing ROS release through both uncoupling and inhibiting succinate dehydrogenase is supported by the finding that both uncouplers and artificial succinate dehydrogenase inhibitors themselves can prevent ischemic cell death

(Ockaili *et al.*, 2001; Holmuhamedov *et al.*, 2004; Rodrigo *et al.*, 2002).

Although we do not believe increases in mitochondrial ROS are involved in cardioprotection promoted by mitoK$_{ATP}$ agonists, there is good support for a role of moderate increases in mitochondrial ROS release acting as signals during ischemic preconditioning (Vanden Hoek *et al.*, 1998; Lebuffe *et al.*, 2003). During preconditioning, changes in local oxygen levels and the activity of respiratory complexes result in increased levels of superoxide radical generation (Vanden Hoek *et al.*, 1998; daSilva *et al.*, 2003), leading to mild oxidative stress and activation of protective pathways. One such pathway is mitoK$_{ATP}$ itself, which is activated by oxidation (Zhang *et al.*, 2001). Thus, mitoK$_{ATP}$ may function in a manner similar to mitochondrial uncoupling proteins, which are also activated by oxidative stress and prevent excessive mitochondrial ROS release (Brand *et al.*, 2004). Indeed, augmented uncoupling protein activity is strongly protective against ischemic damage (Mattiasson *et al.*, 2003; Hoerter *et al.*, 2004).

Together, our results suggest that among the multiple protective roles mitoK$_{ATP}$ opening has during ischemia, a key function is the prevention of Ca^{2+}-promoted oxidative stress, maintaining mitochondrial membrane integrity by preventing MPT, an important cause of post-ischemic cell death (see Fig. 5). This finding further illustrates the central regulatory role of mitochondrial ion transport in the maintenance of cellular integrity.

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