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Reactive oxygen species and permeability transition pore in rat liver and kidney mitoplasts

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Abstract Mitochondrial permeability transition is typically characterized by Ca^{2+} and oxidative stress-induced opening of a nonselective proteinaceous membrane pore sensitive to cyclosporin A, known as the permeability transition pore (PTP). Data from our laboratory provide evidence that the PTP is formed when inner membrane proteins aggregate as a result of disulfide cross-linking caused by thiol oxidation. Here we compared the redox properties between PTP in intact mitochondria and mitoplasts. The rat liver mitoplasts retained less than 5% and 10% of the original outer membrane markers monoamine oxidase and VDAC, respectively. Kidney mitoplasts also showed a partial depletion of hexokinase. In line with the redox nature of the PTP, mitoplasts that were more susceptible to PTP opening than intact mitochondria showed higher rates of H_2O_2 generation and decreased matrix NADPH-dependent antioxidant activity. Mitoplast PTP was also sensitive to the permeability transition inducer *tert*-butyl hydroperoxide and to the inhibitors cyclosporin A, EGTA, ADP, dithiothreitol and catalase. Taken together, these data indicate that, in mitoplasts, PTP exhibits redox regulatory characteristics similar to those described for intact mitochondria.

Keywords Mitochondrial inner membrane · Reactive oxygen species · Permeability transition pore · Calcium · Mitoplast

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Introduction

Mitochondrial permeability transition is a nonselective permeabilization of the inner mitochondrial membrane, typically promoted by oxidative stress and an excessive accumulation of Ca^{2+} in the matrix (Zoratti and Szabò 1995; Crompton 1999; Kowaltowski et al. 2001; Vercesi et al. 2006). Despite extensive research, the exact nature of the membrane modifications that lead to mitochondrial permeability transition still remains unclear; being reversible under certain defined conditions (Vercesi 1984; Al-Nasser and Crompton 1986; Novgorodov et al. 1994; Castilho et al. 1996) and involves membrane protein thiol oxidation by reactive oxygen species (ROS), reactive nitrogen species (RNS) or thiol reagents (Fagian et al. 1990; Lenartowicz et al. 1991; Petronilli et al. 1994; Castilho et al. 1995; Scarlett et al. 1996). The thiol groups of proteins found in the inner membrane are the primary targets for oxidative damage, a process stimulated by excessive accumulation of Ca^{2+} in the matrix (Kowaltowski et al. 2001; Kowaltowski et al. 2009). The oxidation of membrane protein thiols results in the formation of disulfide bonds and protein aggregation, leading to changes in membrane conformation. These changes, in the presence of Ca^{2+}, form a large nonselective pore known as the permeability transition pore (PTP) (Fagian et al. 1990; Kowaltowski et al. 2001).

The protein composition of the PTP is still the subject of debate (Krauskopf et al. 2006; Baines et al. 2007; Basso et al. 2008; Halestrap 2009; Zoratti et al. 2010). Many papers suggest that it is minimally composed of or modulated by the following proteins: the inner membrane proteins adenine nucleotide translocase (Lê Quôc and Lê Quôc 1988; Crompton et al. 1998; Kokoszka et al. 2004) and phosphate carrier (Leung et al. 2008); the matrix protein

cyclophilin D (Crompton et al. 1998; Woodfield et al. 1998; Baines et al. 2005); and the outer membrane proteins VDAC (the voltage-dependent anion channel) (Szabó and Zoratti 1993; Beutner et al. 1996; Crompton et al. 1998; Marzo et al. 1998), the 18 kDa translocator protein (Sileikyte et al. 2011) and possibly hexokinase (Beutner et al. 1996; Marzo et al. 1998). The hypothesis that outer membrane proteins are part of the PTP is in contrast to our previous work and those of other groups who demonstrated that the PTP opening can be observed in mitochondria devoid of the outer membrane (mitoplasts) (Fagian et al. 1990; Szabó and Zoratti 1991; Zhang et al. 2008; Sileikyte et al. 2011) and in submitochondrial particles (Fagian et al. 1990; Grijalba et al. 1999).

In this study we investigated the redox properties of the PTP in rat liver and kidney mitoplasts, which are devoid of more than 90% of outer membrane proteins. The first account of this work was presented at the 39th Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology in 2009 (Ronchi et al. 2009).

Material and methods

Isolation of liver and kidney mitochondria Liver and kidney mitochondria were isolated from adult rats by conventional differential centrifugation (Kaplan and Pedersen 1983; Wallin et al. 1987). Whole kidneys (cortex and medulla) and livers from female Wistar rats were rapidly removed, finely minced and homogenized in 250 mM sucrose, 1 mM EGTA and 10 mM HEPES buffer (pH 7.2). The mitochondrial suspensions were washed twice in the same medium containing 0.3 mM EGTA, and the final pellet was resuspended in an EGTA-free buffer.

Mitoplasts Mitoplasts were prepared according to Schnaitman and Greenawalt (1968) from suspensions of mitochondria isolated from rat liver and kidney with a protein concentration of 90–100 mg/mL. One milliliter of the mitochondrial suspension was added to 1.0 mL of medium containing 225 mM mannitol, 75 mM sucrose, 2% bovine serum albumin, 5 mM HEPES (pH 7.2) and digitonin (2.0% and 1.2% for liver and kidney mitochondria, respectively) followed by gentle stirring in an ice bath for 15 min. The suspension was diluted with 10 mL of the above medium without digitonin and centrifuged for 10 min at 12,000×*g*. The pellet was then resuspended in 5 mL of the same digitonin-free medium and centrifuged again under the same conditions. The mitoplast pellet was washed twice in 5 mL of the same medium, and the final pellet was resuspended in 250 mM sucrose and 5 mM HEPES buffer (pH 7.2). For standardization purposes intact mitochondria used in the experiments were also submitted to this isolation protocol but without digitonin. Digitonin was obtained from Sigma-Aldrich (catalog code D-141).

Protein measurement The protein content of the mitochondrial and mitoplast suspensions were determined using the Biuret assay in the presence of 1% deoxycholate (Gornall et al. 1949) with bovine serum albumin as the standard.

Citrate synthase activity The conversion of oxaloacetate and acetyl-CoA to citrate and SH-CoA catalyzed by citrate synthase was monitored by measuring the colorimetric product thionitrobenzoic acid (Shepherd and Garland, 1969). Mitochondrial or mitoplast fractions (30 μg/mL) were incubated at 37 °C in a buffer containing 50 mM Tris–HCl (pH 8.0), 0.1% Triton X-100, 250 μM oxaloacetate, 50 μM acetyl-CoA, and 100 μM 5,5′-dithiobis(2-nitrobenzoic acid). The increase in absorbance at 412 nm was monitored for 5 min using a Hitachi U-3000 spectrophotometer.

Monoamine oxidase (MAO) activity MAO activity was determined by monitoring for 5 min the increase in absorbance at 250 nm caused by the formation of benzaldehyde from benzylamine oxidation (Tabor et al. 1954). Mitochondrial or mitoplast fractions (0.5 mg/mL) were incubated at 37 °C in a buffer containing 50 mM K_2HPO_4 (pH 7.4), 0.5% Triton X-100 and 1 mM benzylamine.

Hexokinase activity Hexokinase activity was determined using a previously described method with minor modifications (Santiago et al. 2008) by measuring NADPH production at 340 nm for 5 min. The mitochondrial or mitoplast fractions (0.2 mg/mL) were incubated at 37 °C in a buffer containing 20 mM Tris–HCl (pH 7.4), 5 mM glucose, 10 mM $MgCl_2$, Triton X-100 0.5%, 50 μM p1,p5-di(adenosine-5′) pentaphosphate pentasodium salt (Ap5A), 2U/mL glucose-6-phosphate dehydrogenase and 1 mM $NADP^+$. The reactions were initiated by adding 1 mM ATP.

Detection of the levels of VDAC isoforms 1, 2, and 3 by western blotting For this, aliquots of mitochondrial or mitoplast suspensions were used, and the matrix proteins were released by three subsequent freeze/thaw cycles. Samples were centrifuged at 15,000×*g* for 2 min, and the pellets were resuspended and boiled for 3 min under non-reducing conditions [1.25 M sucrose, 250 mM Tris–HCl (pH 7.4), 5% sodium dodecyl sulfate (SDS), 10 mM EDTA and 0.05% bromophenol blue]. Protein concentrations were determined using the Lowry method (1951). Mitochondrial and mitoplast protein lysates (2.5–50 μg) were electrophoresed on 12% SDS-polyacrylamide gels (SDS-PAGE), transferred onto nitrocellulose membranes (Potran, Shleicher & Schuell, USA) and stained with Ponceau S

(Sigma) to verify transfer efficiency. The membranes were blocked with 5% non-fat dry milk in 20 mM Tris–HCl (pH 7.6) containing 150 mM NaCl and 0.1% Tween-20 (TBST) for 16 h and then probed for 2 h at room temperature with anti-VDAC 1 polyclonal antibody (1:200, ab15895, Abcam Inc.), anti-VDAC 2 or 3 polyclonal antibodies (1:200, PA1-958 and PA1-959, respectively, Affinity BioReagents). After washes with TBST, the membranes were incubated with a biotin conjugated secondary antibody (1:1,000) for 1.5 h at 25 °C. The reaction was revealed by colorimetry using the Alkaline Phosphatase Immun-Blot® assay kit (Bio-Rad Laboratories). The membranes were analyzed by a computer after being digitally scanned. Molecular mass markers (PageRuler Prestained Protein Ladder, Fermentas Life Sciences, Hanover, MD, USA) were run at the same time in all gels.

Standard incubation procedure Measurements of mitochondrial and mitoplast oxygen consumption, reactive oxygen species production, membrane potential and swelling were carried out at 28 °C with continuous magnetic stirring in a standard medium containing 125 mM sucrose, 65 mM KCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM HEPES buffer (pH 7.2), ~10 μM contaminant Ca^{2+} and a complex I substrate mixture (2 mM malate, 1 mM pyruvate, 1 mM α-ketoglutarate and 1 mM glutamate). Other additions are indicated in the figure legends.

Oxygen consumption measurements Oxygen consumption was measured using a computer-interfaced Clark-type oxygen electrode from Hansatech Instruments in a sealed glass cuvette.

Measurement of transmembrane electrical potential Mitochondrial and mitoplast membrane potentials were monitored by following the changes in safranine fluorescence, which were recorded on a Shimadzu (RF-5301PC) spectrofluorometer operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 5 nm.

Reactive oxygen species production H_2O_2 production by isolated mitochondria or mitoplasts was monitored by measuring the conversion of Amplex Red to highly fluorescent resorufin in the presence of extramitochondrial horseradish peroxidase. Mitochondrial suspensions were incubated in the presence of 10 μM Amplex Red and 1 U/mL horseradish peroxidase, and fluorescence was monitored over time with a temperature-controlled spectrofluorometer using excitation and emission wavelengths of 563 and 587 nm, respectively, and slit widths of 3 nm. Under these conditions, a linear increment in fluorescence indicates the rate of H_2O_2 released from mitochondria and mitoplasts.

Superoxide ($O_2^{\cdot-}$) production was determined by measuring mitoSOX Red oxidation to fluorescent products

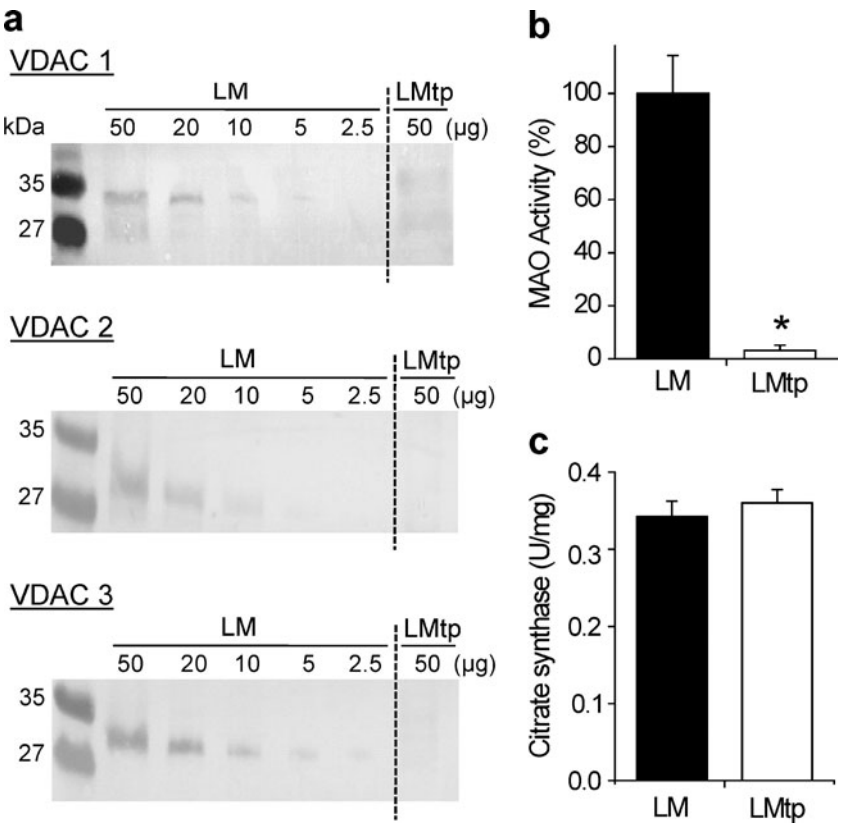

Fig. 1 Characterization of rat liver mitoplasts. **Panel a:** Comparison of VDAC isoform 1, 2 and 3 content in intact rat liver mitochondria (LM) and mitoplasts (LMtp). Mitochondrial or mitoplast membrane proteins (2.5–50 μg) were added to each lane as indicated. **Panel b:** Determination of MAO activity in LM and LMtp. *Significant in relation to LM; $p<0.01$. **Panel c:** Determination of citrate synthase activity in LM and LMtp

(Piacenza et al. 2007) in mitochondrial or mitoplast suspensions supplemented with 1 μM mitoSOX. The amount of fluorescent products generated was measured using excitation and emission wavelengths of 510 and 580 nm, respectively, and slit widths of 10 nm.

Kinetics of pyridine nucleotide oxidation Changes in the redox state of pyridine nucleotides (NAD(P)H) in the mitochondrial or mitoplast suspensions (1 mg/mL) were monitored in a spectrofluorometer using excitation and emission wavelengths of 366 and 450 nm, respectively, and slit widths of 5 nm.

Mitochondrial and mitoplast swelling Swelling was estimated by monitoring the decrease in the turbidity of the mitochondrial or mitoplast suspensions measured at 520 nm using a Hitachi U-3000 spectrophotometer.

Statistical analysis Data from the experiments were analyzed using the unpaired Student's *t*-test. Results are presented as representatives or averages±standard deviation (SD) of at least three experiments with different preparations.

Results and discussion

The experiments depicted in Fig. 1a–c were designed to determine whether the rat liver mitoplast preparations used were devoid of the outer membrane. Figure 1a shows that, in contrast to the intact mitochondria, the mitoplasts obtained were almost completely devoid of isoforms 1, 2 and 3 of the outer membrane protein VDAC, as determined by western blot analysis. The activity of monoamine oxidase (MAO), a mitochondrial protein anchored to the outer membrane (Edmondson et al. 2009), was negligible in the mitoplast suspension compared with the corresponding activity in the intact mitochondria, indicating that the outer membrane was removed from nearly all of the organelles (Fig. 1b). Similar activity was observed in both preparations for citrate synthase, a marker of the mitochondrial matrix, indicating that the amount of organelles in the mitoplast suspension per mg of protein was not significantly different from that in the intact mitochondrial preparation (Fig. 1c).

The respiratory capacity and coupling state of the liver mitoplasts were compared with those for the intact mitochondria in experiments measuring resting and ADP-induced state-3 respiration. Although state-3 respiration rates were much lower in the mitoplasts than in the intact mitochondria (13.4±5.5 *versus* 56.4±9.4 nmol O_2×mg protein^{-1}×min^{-1}), the inclusion of 5 μM cytochrome *c* in the mitoplast preparation

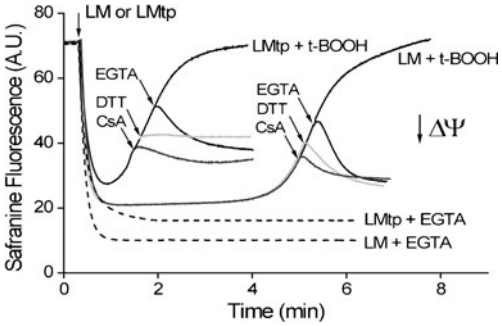

Fig. 2 Effect of *tert*-butyl hydroperoxide plus Ca^{2+} on the membrane potential of liver mitochondria and mitoplasts. The arrows show where LM or LMtp (0.5 mg/mL) were added to the incubation medium, which contained 200 μM EGTA or 10 μM Ca^{2+} plus 300 μM *tert*-butyl hydroperoxide (*t*-BOOH). The arrows show where the PTP inhibitors cyclosporin A (CsA; 1 μM), 3 mM dithiothreitol (DTT) or 1 mM EGTA were added to the experiments

partially restored the state-3 respiration rate (31.8±9.8 nmol O_2×mg protein^{-1}×min^{-1}). However, the respiratory control increased only slightly and remained much lower in the mitoplasts than in the intact mitochondria (2.9±0.56 *versus* 6.9±1.01). The respiration rates of the intact mitochondria were not significantly altered by the addition of 5 μM cytochrome *c*. Except for the experiments measuring ROS production, all further experiments evaluating PTP opening in mitoplasts described in this paper were conducted in the presence of cytochrome *c*.

For comparison of the redox PTP properties in intact mitochondria and mitoplasts experiments were performed to test the reversibility of ΔΨ changes associated with PTP opening and closing using permeability transition inducers and inhibitors. Figure 2 shows that the prooxidant *tert*-butyl hydroperoxide plus Ca^{2+} stimulated PTP

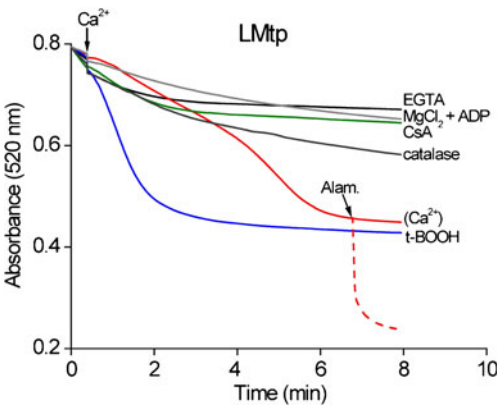

Fig. 3 Estimation of PTP opening in liver mitoplasts (LMtp) based on swelling measurements. LMtp (0.5 mg/mL) were incubated in medium containing 200 μM EGTA, 2 mM $MgCl_2$ plus 300 μM ADP, 1 μM CsA, 2 μM catalase or 300 μM*t*-BOOH, as indicated. Ca^{2+} (10 μM) was added to all experiments where indicated. Alamethicin (Alam.; 20 μg/mL) was added to some experiments to ensure maximum organelle swelling

opening in both preparations (indicated by the decrease in the lag between the addition of mitochondria or mitoplasts to the reaction medium and the sharp decrease in the $\Delta\Psi$). Mitoplasts were more susceptible than mitochondria to *tert*-butyl hydroperoxide plus Ca^{2+}-induced PTP. In contrast, the PTP inhibitors EGTA, cyclosporin A and dithiothreitol blocked the decrease in $\Delta\Psi$ and in some cases reversed it.

The opening of the PTP in liver mitoplasts was also confirmed by swelling experiments (Fig. 3). Calcium-induced swelling in liver mitoplasts was inhibited by either EGTA, catalase, ADP plus $MgCl_2$ or cyclosporin A, while the prooxidant *tert*-butyl hydroperoxide stimulated organelle swelling. The addition of the pore-forming compound alamethicin (He et al. 1996) maximized organelle swelling and thus caused a rapid drop in absorbance. These results indicate that mitoplasts and intact mitochondria have similar responses to known PTP inducers and inhibitors. This characterization of mitoplast PTP regulation adds new information to previous reports in the literature (Fagian et al. 1990; Zhang et al. 2008) describing the occurrence of Ca^{2+}-induced swelling and Ca^{2+} release in mitoplasts obtained from digitonin-treated mitochondria. In addition, Sileikyte et al. (2011) recently reported the occurrence of cyclosporin A-sensitive PTP in rat liver mitoplasts. Regulation of the PTP by the 18 kDa outer membrane translocator protein was also demonstrated (Sileikyte et al. 2011).

The results shown in Figs. 1, 2, and 3 indicate that VDAC isoforms are not necessary for PTP opening. These findings are in accord with recent reports using liver and fibroblast mitochondria deficient in VDAC isoforms (Krauskopf et al., 2006; Baines et al., 2007). In addition, the results obtained with mitoplasts are more consistent

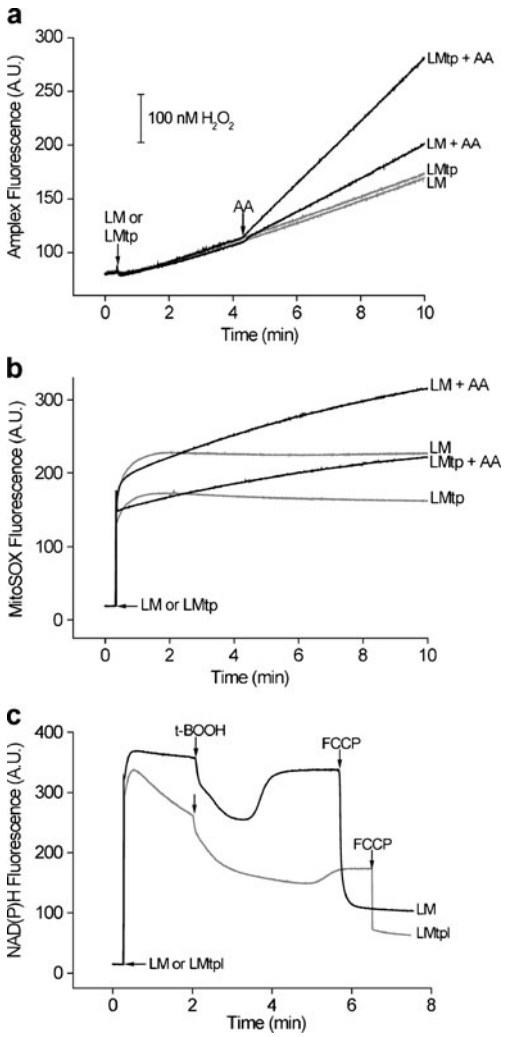

Fig. 4 Measurement of reactive oxygen species generation and NAD(P)H redox state in liver mitochondria and mitoplasts. **Panel a:** Effect of antimycin A on H_2O_2 production. LM or LMtp (0.25 mg/mL) were incubated in reaction medium containing Amplex Red plus horseradish peroxidase. Where indicated by an arrow, 2 μM antimycin A (AA) was added. **Panel b:** Effect of antimycin A on superoxide production. LM or LMtp (0.25 mg/mL) were incubated in reaction medium containing mitoSOX Red in the presence or absence of antimycin A as indicated in the figure. **Panel c:** Measurements of NAD(P)H redox state in LM and LMtp. The arrows show where the prooxidant *t*-BOOH (25 μM) and the protonophore FCCP (0.5 μM) were added to induce NAD(P)H oxidation

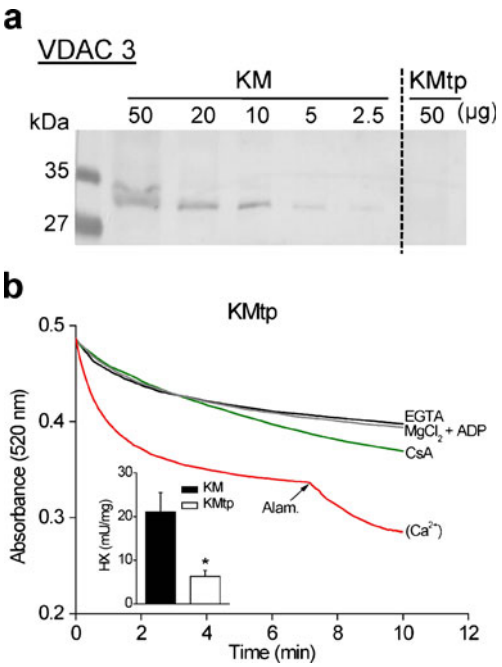

Fig. 5 Estimation of PTP opening in kidney mitoplasts (KMtp) based on swelling measurements. **Panel a:** Comparison of the VDAC isoform 3 content in intact kidney mitochondria (KM) and mitoplasts (KMtp). Mitochondrial or mitoplast membrane proteins (2.5–50 μg) were added to each lane as indicated. **Panel b:** KMtp (0.5 mg/mL) were incubated in medium containing 10 μM Ca^{2+} in the presence of 200 μM EGTA, 2 mM $MgCl_2$ plus 300 μM ADP or 1 μM CsA, as indicated. Alamethicin (Alam.; 20 μg/mL) was added where indicated by the arrow. ***Figure inset:*** Determination of hexokinase activity (HX) in KM and KMtp. *Significant in relation to KM; $p<0.01$

than those obtained with VDAC-deficient cells because mitoplasts are not susceptible to compensatory adaptations. Compensatory adaptations have been described in knockout studies of mitochondrial genes where organisms have shown significant changes in the expression of other functionally related genes (Lee et al., 2008; Sutak et al., 2008).

The next experiments evaluated H_2O_2 (Fig. 4a) and superoxide production (Fig. 4b) and NAD(P)H redox state (Fig. 4c) in intact mitochondria and mitoplasts. Antimycin A was added to the experiments to enhance reactive oxygen species generation at the respiratory chain complex III (Kowaltowski et al. 2009). Under these experimental conditions, mitoplasts showed higher rates of H_2O_2 generation than mitochondria (Fig. 4a). Interestingly, superoxide generation was similar in both preparations (Fig. 4b). In addition, it was observed that mitoplasts do not sustain NAD(P)H fluorescence as intact mitochondria and that when challenged by *tert*-butyl hydroperoxide addition (Lötscher et al., 1979) showed a slower recovery in fluorescence (Fig. 4c). These results indicate that although mitochondria and mitoplasts generate similar amounts of superoxide, the latter possesses less H_2O_2 detoxifying activity, explaining the higher mitoplast susceptibility to PTP opening.

Because rat liver mitochondria have low concentrations of hexokinase (Santiago et al. 2008), kidney mitochondria were used to prepare mitoplasts in order to evaluate the role of hexokinase in PTP. Depletion of the outer membrane of kidney mitochondria was estimated by VDAC 3 expression. Kidney mitoplasts were found to retain less than 10% of the VDAC 3 content (Fig. 5a) and only 27% of the hexokinase activity (Fig. 5b, *inset*) of intact kidney mitochondria. These data indicate that kidney mitoplasts are adequate for investigating the possible participation of outer membrane proteins, including hexokinase, in PTP assembly. As shown for liver mitoplasts (Fig. 3), kidney mitoplasts swelled in the presence of Ca^{2+} in a process that was sensitive to cyclosporin A, EGTA, and ADP plus Mg^{2+} (Fig. 5b).

Thus, the data presented here is in accordance with the concept that the inner mitochondrial membrane and the matrix contain all the components required for PTP assembly. The PTP in mitoplasts showed redox regulatory characteristics similar to those described for intact mitochondria. In addition, mitoplasts are more susceptible to PTP opening due to its lower capacity to sustaining a reduced state of NADPH.

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