On the properties of calcium-induced permeability transition in neonatal heart mitochondria

Natalia Pavón · Juan Carlos Gallardo · Luz María Hernández-Esquivel ·
Mohammed El-Hafidi · Mabel Buelna-Chontal · Cecilia Zazueta ·
Sara Rodríguez-Enríquez · Edmundo Chávez

Abstract Permeability transition was examined in heart mitochondria isolated from neonate rats. We found that these mitochondria were more susceptible to Ca^{2+}-induced membrane leakiness than mitochondria from adult rats. In K^+ containing medium, at 25 °C, mitochondria were unable to accumulate Ca^{2+}. Conversely, in Na^+ containing medium, mitochondria accumulated effectively Ca^{2+}. At 15 °C mitochondria accumulated Ca^{2+} regardless of the presence of K^+. Kinetics of Ca^{2+} accumulation showed a similar Vmax as that of adult mitochondria. Lipid milieu of inner membrane contained more unsaturated fatty acids than adult mitochondria. Aconitase inhibition and high thiobarbituric acid-reactive substances (TBARS) indicate that oxidative stress caused mitochondrial damage. In addition, proteomics analysis showed that there is a considerable diminution of succinate dehydrogenase C and subunit 4 of cytochrome oxidase in neonate mitochondria. Our proposal is that dysfunction of the respiratory chain makes neonate mitochondria more susceptible to damage by oxidative stress.

Keywords Neonate mitochondria · Permeability transition · Calcium · Oxidative stress · Heart mitochondria · Mitochondrial proteomics

N. Pavón · J. C. Gallardo · L. M. Hernández-Esquivel ·
M. Buelna-Chontal · C. Zazueta · S. Rodríguez-Enríquez ·
E. Chávez (✉)
Departamento de Bioquímica,
Instituto Nacional de Cardiología, Ignacio Chávez,
Juan Badiano # 1, Tlalpam,
Mexico, DF 014080, Mexico
e-mail: echavez@salud.gob.mx

M. El-Hafidi
Departamento de Biomedicina, Cardiovascular,
Instituto Nacional de Cardiología, Ignacio Chávez,
Mexico, DF 014080, Mexico

Introduction

Mitochondria undergo membrane permeability transition through the opening of a non-specific channel of a diameter of at least 2.8 nm that allows the release of matrix metabolites (Zoratti and Szabó 1995; Zoratti et al. 2005). Activation of this transmembrane pathway requires matrix Ca^{2+} overload in addition to an inducing agent (Gunter and Pfeiffer 1990). Among the wide variety of inducers, carboxyatractyloside has been used successfully and extensively to promote permeability transition. The latter observation led to the conclusion that adenine nucleotide translocase (ANT) plays a central role in mitochondrial Ca^{2+} homeostasis (Halestrap, and Davidson 1990). Such a proposal is strengthened by the fact that the unspecific membrane leakiness can be suppressed by adenine nucleotides, being ADP the most effective (Haworth and Hunter 2000). Modulation of the increased permeability has been associated with the orientation of ANT across the inner membrane. In this context, it has been claimed that the mechanism by which carboxyatractyloside (CAT) induces calcium release is through the fixing of ANT to the cytosolic side of the inner membrane. Conversely, ADP inhibits pore opening because it locks ANT into the matrix side. Such a conformational change must be strictly dependent on membrane fluidity, which in turn depends on its lipid composition. Previously, we have reported that mitochondria from brown adipose tissue are more resistant than kidney mitochondria to CAT-induced permeability transition due to the high membrane concentration of cardiolipin, as compared with kidney mitochondria (Chávez et al. 1996). With respect to the mechanism underlying the development of the Ca^{2+}-induced pore opening, a number of authors have considered Ca^{2+}-dependent oxidative stress as its trigger (Brookes et al. 2004;

Petrosillo et al. 2004; Peng and Jou 2010; Adam-Vizi and Starkov 2010).

A wide sort of mitochondrial sources have been used to contribute to the extensive knowledge about the properties of membrane permeability transition, i.e., from yeast, parasites, and mammals like rats, among others. However, only a few studies on permeability transition have been done using mitochondria isolated from neonate rats, i.e. Meriova et al. (2010). Therefore, we used heart mitochondria from 7-day-old rats, keeping in mind the following questions: Does permeability transition occur as in other types of mitochondria? If so, what are the characteristics? The purpose of the present work was to attempt to solve these questions. The results were compared with those obtained with adult heart mitochondria. It is shown that when newborn mitochondria were incubated in medium containing KCl, the addition of Ca^{2+} induced membrane leakage; the opposite occurred in medium containing NaCl. In a K^+ medium, Ca^{2+} effectively remained accumulated when the incubation temperature was maintained at 15 °C. Kinetic study of Ca^{2+} uptake demonstrated a high V_{max} in adult mitochondria and in newborn mitochondria, with no change in $K_{0.5}$. Analysis of fatty acids content within the inner membrane showed a higher amount of unsaturated fatty acids in neonate mitochondria than in adult mitochondria. In concordance with the above, the amount of thiobarbituric acid-reactive substances (TBARS) was elevated in neonate as compared to adult mitochondria. Oxidative stress in newborn mitochondria was also demonstrated through the observed inhibition of aconitase activity. As a whole, these results provide support to our proposal that the increased sensitivity to Ca^{2+}-induced permeability transition shown by neonate mitochondria should be due to an oxidative stress having as substrate the high amount of membrane unsaturated fatty acids. Further, the diminished activity of superoxide dismutase as well as the low expression of succinate dehydrogenase and subunit 4 of cytochrome oxidase, found in neonate mitochondria, must be taken into account, since the latter increases the production of reactive oxygen-derived species.

Materials and methods

Heart mitochondria were prepared from neonate rats, 7-day-old, and from adult rats, 60-day-old, by homogenizing the tissue in 0.25 M sucrose–1 mM EDTA, adjusted to pH 7.3 with Tris-base. Submitochondrial particles were prepared after sonication of mitochondria and further centrifugation at $100,000\,g$. Protein was determined by the method of Lowry et al. (1951). Calcium movement was followed by dual spectrometry

at 675–685 nm with Arsenazo III as indicator (Scarpa et al. 1978). Alternatively, calcium accumulation was assayed by using $^{45}Ca^{2+}$ (specific activity 1,020 cpm/nmol) following the Millipore filtration technique; intramitochondrial Ca^{2+} was determined by the radioactivity retained in the filter, using a scintillation counter. Transmembrane electric gradient was analyzed spectrophotometrically at 525–575 nm using the dye safranine. Aconitase activity was analyzed according to Hausladen and Fridovich (1994) as follows: mitochondrial protein was solubilized by adding 0.05% Triton X-100 containing 25 mM phosphate, pH 7.2, followed by the addition of 0.6 mM manganese sulphate, 1 mM citrate, and 0.1 mM NADP. The formed *cis*-aconitate was measured spectrophotometrically at 240 nm. Membrane lipid peroxidation was determined spectrophotometrically as the concentration of TBARS. Superoxide dismutase enzymatic activity was determined in mitochondria by non-denaturating, 8% acrylamide, gel electrophoresis and nitro blue tetrazolium staining as described by Pérez-Torres et al. (2009). Fatty acids constitution of the inner membrane was analyzed in submitochondrial particles (SMP); briefly, 1 mg of SMP was used for the extraction of lipids, according to the method of Folch et al. (1957), in the presence of 50 μg heptadecanoic acid as internal standard. The lipid fractions were transesterified to their fatty acid methyl esters by heating at 80 °C. Fatty acids methyl esters were separated and identified by gas liquid chromatography. The analysis was carried out at 195 °C, using helium gas as carrier, at a flow rate of 2.5 ml/min, as described by El Haffidi et al. (2001). Proteomics analysis of the inner membrane was carried out as described by Rodríguez-Enríquez et al. (2008) briefly, 50 μg of mitochondrial protein were separated onto 12.5% SDS-PAGE and transferred to a PVDF membrane. Afterwards, membranes were incubated with different mouse monoclonal (ATPase, ND1, COX-IV, Glutaminase, PDH-E1α; dilutions 1:1000), rabbit or goat polyclonal (SDHC, ANT, 2-OGDH, dilutions 1:500) antibodies overnight at 4 °C. After washing the membranes, they were incubated with a horse-radish peroxidase conjugated, mouse, rabbit, or goat secondary antibody (dilution 1:2000) and developed in a darkroom using enhanced chemoluminescence. Densitometric analysis was performed using the Scion Image Software, and normalized against its respective load control (in this case ND1), which corresponded to 100% intensity. The basic incubation medium contained 125 mM KCl; 10 mM succinate; 3 mM phosphate; 10 mM HEPES, the mixture was adjusted to pH 7.3. The results are representative of at least three different experiments. Other experimental conditions were as described in the legends of the corresponding figures.

Results

Mitochondria have the ability to accumulate high amount of Ca^{2+} in response to a transmembrane potential generated by the oxidation of substrates by the respiratory chain. Figure 1a depicts Ca^{2+} movement in heart mitochondria isolated from 7-day-old rats, incubated in a medium containing succinate as the substrate and 125 mM KCl as osmotic support. As illustrated in trace a, after a short Ca^{2+} accumulation phase, a fast discharge reaction of the cation took place. This failure of neonate mitochondria to retain Ca^{2+} could be due to the opening of the permeability transition pore. To ascertain the above, cyclosporin A (CSA), a specific inhibitor of the pore, was included in the incubation mixture. As shown in trace b, CSA effectively inhibited Ca^{2+} efflux. The latter indicates that, certainly, Ca^{2+} release can be ascribed to the opening of the non-specific pore. On the other hand, in an early report we showed that the opening of the transition pore closely depends on the incubation temperature (Chávez and Osornio 1988). Figure 1b displays the results of experiments in which neonate mitochondria were incubated at 15 °C. The result in trace a seems to agree with previous data, indicating that at low

temperature Ca^{2+} was accumulated and retained inside the matrix. Further, trace b shows that, at this temperature, carboxyatractyloside (CAT), an inducer of pore opening, slightly activated Ca^{2+} release. The experiment in Fig. 1c was carried out to make a comparison with the accumulation of Ca^{2+} in mitochondria isolated from adult rats. As illustrated, in these mitochondria, the cation remained accumulated, even at an incubation temperature of 30 °C.

Incubation of mitochondria with Ca^{2+} and malate/glutamate, as the oxidizable substrates, promotes the generation of reactive oxygen derived species (ROS) by complex I of the respiratory chain (Bernardi 1999; García et al. 2005a, b). The latter leads to the opening of the transition pore. The experimental results shown in Figure 2a indicate that the oxidation of malate/glutamate did not support Ca^{2+} accumulation, notwithstanding the addition of CSA the divalent cation remained outside mitochondria from newborn rats. This reaction took place only after the addition of succinate. Figure 2b illustrates that, in adult mitochondria, the oxidation of substrates for complex I effectively promoted Ca^{2+} uptake; although, the accumulation rate was certainly faster after the addition of succinate.
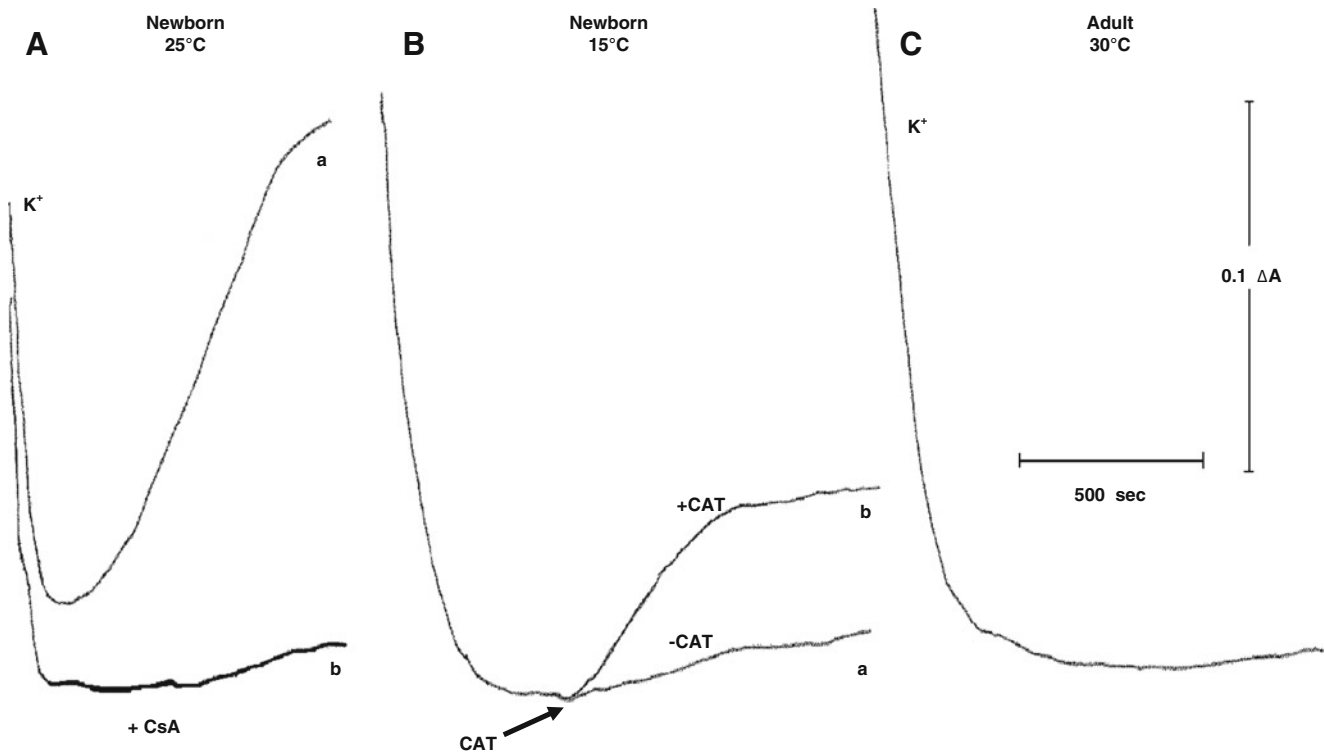
Fig. 1 Effect of temperature on mitochondrial Ca^{2+} movements in neonate and adult mitochondria. In Panel **a**, mitochondria (1.3 mg protein) from neonate rats were incubated at 25 °C, in 3 ml of basic medium, described under Materials and methods. In trace b, 0.5 μM cyclosporin A (CSA) was added. Where indicated in Panel **b**, neonate mitochondri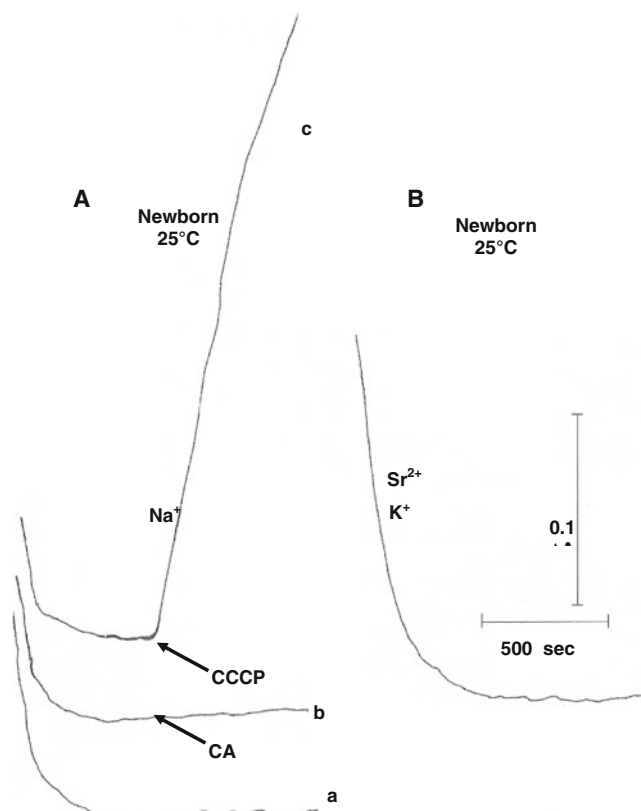a (1.3 mg protein) were incubated at 15 °C. In trace b, 1 μM carboxyatractyloside (CAT) was added. Panel **c** shows Ca^{2+} uptake by mitochondria (1.3 mg protein) from adult rats incubated at 30 °C
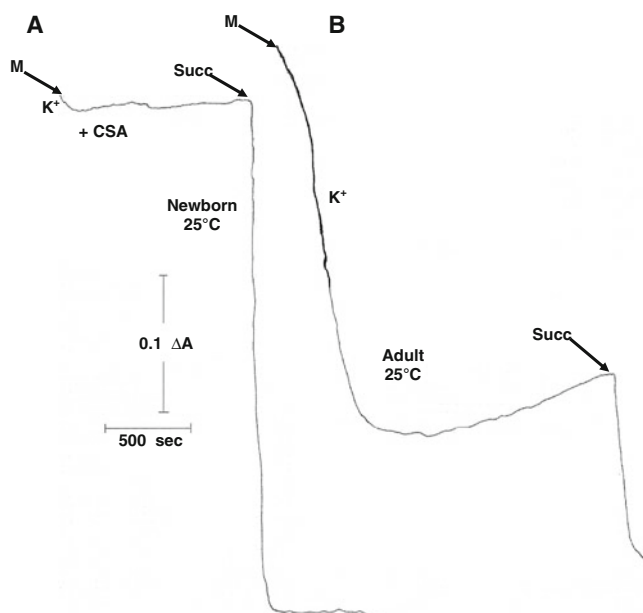
Fig. 2 Calcium movements in heart mitochondria isolated from neonate and adult rats using malate-glutamate as the substrates. Experimental conditions were similar to those described for Fig. 1, with the exception that the 10 mM malate and 10 mM glutamate were used instead of succinate. In Panel **a**, 1.3 mg of neonate mitochondrial protein was added to the incubation mixture, and as indicated the medium contained 0.5 μM CSA plus 5 mM succinate. Panel **b** shows Ca^{2+} accumulation by adult mitochondria (1.3 mg protein). 5 mM succinate was added where indicated. Incubation temperature 25 °C

Fig. 3 Calcium accumulation by neonate mitochondria incubated in sodium containing medium. In Panel **a** and **b**, the experimental conditions were similar to those described for Fig. 1. Except that the incubation mixture contained 125 mM NaCl instead of KCl. In trace b, Panel A, 1 μM CAT was added, and, in trace c, 0.5 μM CCCP was added; control is shown in trace a. In Panel **c**, the medium contained 50 μM $SrCl_2$ instead of 50 μM $CaCl_2$. Temperature 25 °C

Previously, we published a work indicating that in mitochondria incubated in a Na^+ medium, instead of K^+ medium, permeability transition does not occur, even after the addition of Ca^{2+} plus CAT (García et al. 2006). Considering the above, we decided to explore the possibility that in neonate mitochondria, incubated in Na^+ medium, Ca^{2+} would remain accumulated inside the matrix. Figure 3a, trace a, shows that, indeed, neonate mitochondria retained Ca^{2+} when incubated in Na^+ mixture; further, as observed in trace b, CAT did not induce Ca^{2+} discharge; the latter occurred after the addition of the uncoupler carbonyl cyanide-3-chlorophenylhydrazone (CCCP), trace c. Mitochondria accumulate Sr^{2+} through the same way as they accumulate Ca^{2+} (Carafoli 1965; Igbavboa and Pfeiffer 1991; Uribe et al. 1994); however, notwithstanding that Sr^{2+} can substitute Ca^{2+} in several mitochondrial Ca^{2+}-dependent reactions (Caplan and Carafoli 1965; McCormack and Denton 1980; Wernette et al. 1981), it is not able to stimulate permeability transition (Zazueta et al. 2010). Nevertheless, we analyzed the possibility that, in neonate mitochondria, Sr^{2+} could open the pore. Figure 3b shows that, even in a K^+ medium, neonate mitochondria had the ability to accumulate Sr^{2+}.

Figure 4a, trace a, shows the effect of Ca^{2+} on the magnitude of transmembrane potential in neonate mito-

chondria incubated in a K^+ medium. In these conditions, the addition of Ca^{2+} promoted a fast membrane de-energization, after a short lag period. This result contrasts with that obtained with mitochondria incubated in Na^+ medium; trace b shows that a high level of membrane potential was maintained despite the addition of Ca^{2+}. Figure 4b shows that heart mitochondria from adult rats preserve an elevated value of the transmembrane electrical gradient regardless of Ca^{2+} addition; membrane de-energization was attained after CCCP addition.

To assess quantitatively the amount of accumulated Ca^{2+}, as well as to know the kinetics of such a reaction, in neonate and adult mitochondria, the experiment shown in Fig. 5 was performed. The results shown in a Lineweaver-Burk plot indicate, for neonate and adult mitochondria, a similar $K_{0.5}$ for calcium transport: 166 μM as well as similar Vmax, i.e., 164 nmol min^{-1} mg^{-1}.
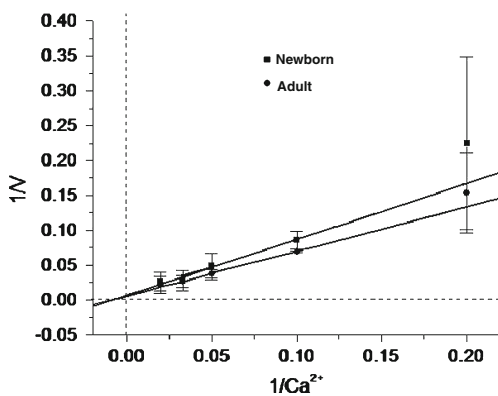
Considering that at the low temperature of 15 °C, Ca^{2+} was effectively accumulated in neonate mitochondria, we examined the membrane fatty acid composition in the inner membrane. The results shown in Table 1 indicate that,
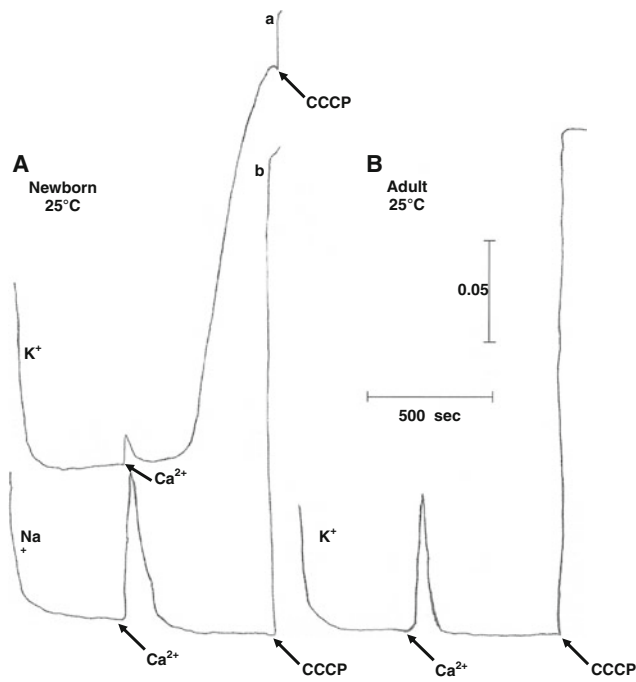
Fig. 4 Effect of sodium and potassium on the transmembrane electrical gradient in neonate mitochondria. Experimental conditions are similar to those described for Fig. 1 except that the medium contained 10 μM safranine instead of Arsenazo III. In Panel **a**, trace a, the incubation medium contained 125 KCl, in trace b the medium contained 125 mM NaCl. Where indicated in trace a and b, 50 μM Ca^{2+} and 0.5 μM CCCP were added. In Panel **b**, adult mitochondria (1.3 mg protein) were incubated in KCl-containing medium, and where indicated 50 μM Ca^{2+} and 0.5 μM CCCP were added. Temperature 25 °C

Table 1 Fatty acids composition of the inner membrane

Fatty acid	Adults	7-day-old
Palmitic	99.3±21.3	140.7±27.3
Palmitoleic	0.7±0.1	1.0±0.2
Estearic	137.9±15.8	149.9±29.9
Oleic	31.2±6.7	48.2±5.9
Linoleic	59.1±4.3	36.7±3.5
g-linolenic	1.8±0	1.5±0.4
a-linoleic	0.9±0.3	1.1±0.1
Arachidic	10.5±0.2	7.7±2.0
Dihomo-g-linoleic	2.9±0.4	7.1±0.9*
Arachidonic	54.8±1.2	107.3±7.3*
Eicosapentaenoic	3.1±0.7	3.4±0.4
Docosapentaenoic	6.1±1.3	10.4±0.1
Docosahexaenoic	46.5±3.1	48.8±0.4
ΣSaturated	247.6±37.3	298.4±59.2
ΣMonounsaturated	31.8±6.6	49.2±5.7*
ΣPolyunsaturated (n-6)	118.6±3.5	152.6±9.6*
ΣPolyunsaturated (n-3)	56.6±4.7	63.8±0.7

Experimental conditions were as described under Materials and methods. The values are expressed in nmol/mg protein and correspond to the mean ± SE ($n=2$ for adults and $n=4$ for 7-day-old). *$P<0.05$ adults vs. 7-day-old

although in neonate mitochondria there is a high amount of palmitic and estearic acids, there is a high concentration of polyunsaturated fatty acids.

The failure to retain Ca^{2+} observed in neonate mitochondria would rest on the increased membrane fluidity due to the high concentration of polyunsaturated fatty acids. However, the existence of double bonds turns the membrane more susceptible to lipid peroxidation. To assess the latter, we measured the reactive species to thiobarbituric acid. Figure 6 indicates that, indeed, after the addition of Ca^{2+} an increased amount of TBARS was generated in mitochondria from newborn rats as compared to that
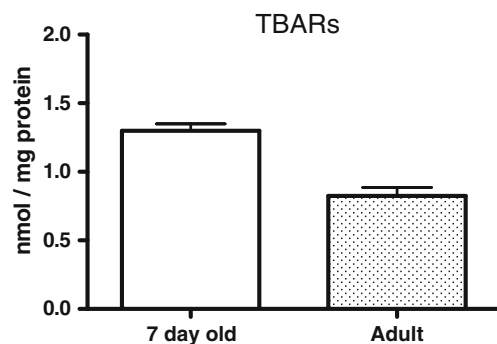

Fig. 5 Lineweaver-Burke plot for the Ca^{2+} uptake in neonate and adult mitochondria. Protein from neonate and adult mitochondria (0.43 mg) was incubated during 1 min in 1 ml of basic medium containing increasing concentrations of $^{45}Ca^{2+}$. Accumulation of the cation was determined as indicated under Materials and methods

Fig. 6 Evaluation of Ca^{2+}-induced oxidative stress in neonate and adult mitochondria. TBARS amount was determined by incubating mitochondria (2 mg protein) in 0.1 ml of basic medium during 30 min. Then, 1.0 ml of 20% acetic acid and 0.8% 2-thiobarbituric acid were added. The mixture was then heated in boiling water for 45 min. After cooling, TBARs were extracted into 2 ml n-butanol. After centrifugation, the butanol layer was measured at 532 nm. A standard curve of MDA was prepared with 1,3,3,3-tetraetoxypropane. The values represent the average ± SD of six different determinations ($P=0.0039$). Analysis was performed with unpaired t-Student

observed in adult mitochondria. As a consequence of the latter, the membrane is most likely to become more leaky.

Besides the analysis of TBARS concentration, aconitase activity resulted as an appropriate marker to assess the damage by oxidative stress, as induced by the superoxide formed on the matrix side of complex III (Muller et al. 2004; García et al. 2006). Therefore, the activity of this enzyme was measured after bringing about mitochondrial oxidative stress. Table 2 shows that, in neonate mitochondria, Ca^{2+}-induced oxidative stress promoted inhibition of the enzyme by around 50%.

Increased oxidative stress in neonate mitochondria would be linked to a diminution in the activity of the oxyradical-scavenging system. To assert the above, the activity of mitochondrial superoxide dismutase was analyzed. Figure 7 illustrates that, indeed, a diminution of around 70% in the activity of SOD was observed in neonate mitochondria, in comparison to that shown by adult mitochondria.

Dysfunction of the respiratory chain has also been involved in the overproduction of reactive oxygen species. Ishii et al. (2011) have shown that in the mev-1 mice model, with mutations in subunits B and C of succinate dehydrogenase, there is an increased production of superoxide anion. In addition, studies on the levels of enzyme activities involved in oxidative phosphorylation have shown different patterns between neonate and adult mitochondria (Marín-García et al. 1997; Drahota et al. 2005; Petrosillo et al. 2008). Thus, a western blot analysis was performed aimed at visualizing, in both classes of mitochondria, differences in the expression of membrane proteins. Figure 8, Panel A, illustrates important differences: in newborn mitochondria there is a considerable diminution in the expression of succinate dehydrogenase C, as well as in the expression of the subunit 4 of cytochrome oxidase and of the 51 kDa subunit of ATPase. In Panel B it is illustrated the densitometry analysis of the western blot.

Discussion

In agreement with the literature, in the field, mitochondria from neonatal rats behave differently, in several parameters,

Table 2 Aconitase activity

nmol *cis*-aconitate/min/mg	
Adult	138±24
	n=7
7-day-old	*68±7.5
	n=7

Inhibition of the aconitase activity in mitochondria from neonate rats in comparison to that observed in mitochondria from adult rats. The activity of the enzyme was determined in 150 μg protein as described under Materials and methods. Values are mean ± SD of seven different mitochondrial preparations.*$P<0.001$

from that observed in adult mitochondria, i.e., respiratory rate, Ca^{2+} homeostasis, and the expression of membrane proteins (Marín-García et al. 1997; Drahota et al. 2005; Petrosillo et al. 2008; Haghberg 2004). In this work, we studied the effects of the cationic composition of the incubation medium, as well as of the lipid composition of the inner membrane on the ability of Ca^{2+}, in combination to oxidative stress, to promote membrane leakage in newborn mitochondria. The initial results cover two contrasting aspects on membrane permeability transition, both of them related with the effect of K^+ and Na^+ on this process. On the one hand, it was shown that K^+ confers a high sensitivity to the neonate mitochondrial membrane to switch from a selective permeable to a hyperpermeable state, after the addition of Ca^{2+}. Besides, it was demonstrated that Na^+ renders mitochondria resistant to undergo pore opening after Ca^{2+} addition. Concerning K^+, it should be mentioned that this cation attains concentrations up to 130 mM (Kauffman et al. 1980) in mitochondria. This amount of K^+ may have a screening effect on negative charges supplied by membrane proteins and phospholipids, as well as by matrix anions. A diminution in the density of non-specific negative charges would increase the free matrix Ca^{2+} concentration required to stimulate pore opening, provided it binds to a target site. It must be considered that a critical level of exchangeable Ca^{2+} is required to trigger membrane permeability transition, i.e., 20 nmol/mg (Chávez et al. 1991).

In contrast are the observations made in Na^+-incubated mitochondria, i.e., Ca^{2+} remained accumulated and a high level of transmembrane electrical gradient was sustained. In order to explain the protective effect conferred by Na^+ to avoid permeability transition, we must take into account that this cation has been used to deplete mitochondria from matrix K^+ (Gómez-Puyou et al. 1969; Chávez et al. 1991; García et al. 2006). It should be noted that the loss of K^+ is not replaced by Na^+ due to the fast Na_{in}^+/H_{out}^+ exchange reaction (Brierley et al. 1977; Bernardi 1999). A diminution in matrix K^+ increases the nonspecific negative sites for Ca^{2+}, which, in turn, diminishes the free Ca^{2+} concentration required to induce pore opening. On the other hand, we found that Sr^{2+}, with a similar ionic radius to Ca^{2+}, i.e., 1.12 Å and 0.99 Å, respectively, was effectively accumulated by neonatal mitochondria. This result strengthens the proposal about the existence of specific sites for Ca^{2+}. To this regard, it has been suggested that cardiolipin, the phospholipid that is part of the annulus of ANT, would be the target site for Ca^{2+} (Petrosillo et al. 2010). Paradies et al. (2009) have discussed that Ca^{2+}, at high concentrations, induces cardiolipin oxidation, which, in turn, triggers mitochondrial permeability transition. We do not rule out that Ca^{2+} binding sites could involve membrane proteins other than

Fig. 7 Superoxide dismutase activity in mitochondria isolated from neonate (**a**) and adult (**b**) rats. Experimental conditions as described under Materials and methods. In **c**, the standard activity is shown. Charge control was evaluated in the presence of adenine nucleotide translocase (ANT). The values represent the average ± SD of five different determinations. $P < 0.001$
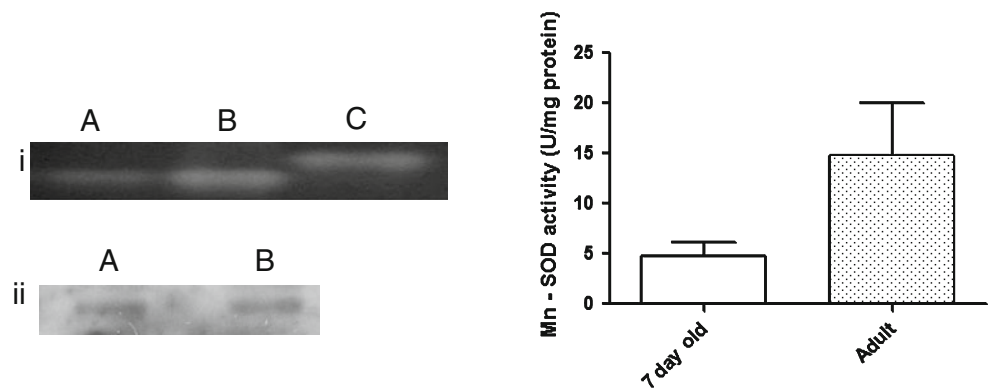
ANT, i.e., the phosphate carrier, as has been proposed by Leung et al. (2008) and Gutierrez-Aguilar et al. (2010).

Another important issue related with permeability transition was the finding that this reaction was enhanced or reduced, depending on the incubation temperature. Concerning the latter, Qian et al. (2004) have shown that heat stress induces membrane permeability transition; in addition, Leduck et al. (1998) showed that permeability transition is associated with changes in temperature. On the same token, we have shown that permeability transition as induced by carboxyatractyloside or agaric acid is highly dependent on the incubation temperature (García et al. 2005a, b). The temperature dependence of the pore closed/open cycles point to membrane fluidity. The above, in turn, should be closely related with the proportion of membrane unsaturated fatty acids. Indeed, as was demonstrated these species of acids are in a higher proportion in newborn mitochondria than in adult mitochondria.

As discussed by Peng and Jou (2010), Ca^{2+} supraload leads to stimulation for ROS generation by inducing cytochrome c dissociation. This reaction produces a partial inhibition in the respiratory chain; thus the electrons are deflected from Complex III towards oxygen generating superoxide anion. The susceptibility of membranes, from neonate mitochondria, to be oxidized by ROS was shown by the experiment in which TBARS were increased. The high concentration of unsaturated fatty acids found in such mitochondria appeared to be responsible for the increase. It is provocative to assume that among the wide diversity of fatty acids vulnerable to oxidation would be those located on cardiolipin. As known, cardiolipin peroxidation underlies one of the multiple triggers of permeability transition (Petrosillo et al. 2004; Petrosillo et al. 2008). Aside from lipid peroxidation, inhibition of the aconitase enzyme was observed; this enzyme has been used as a marker of mitochondrial oxidative injury (Hausladen and Fridovich
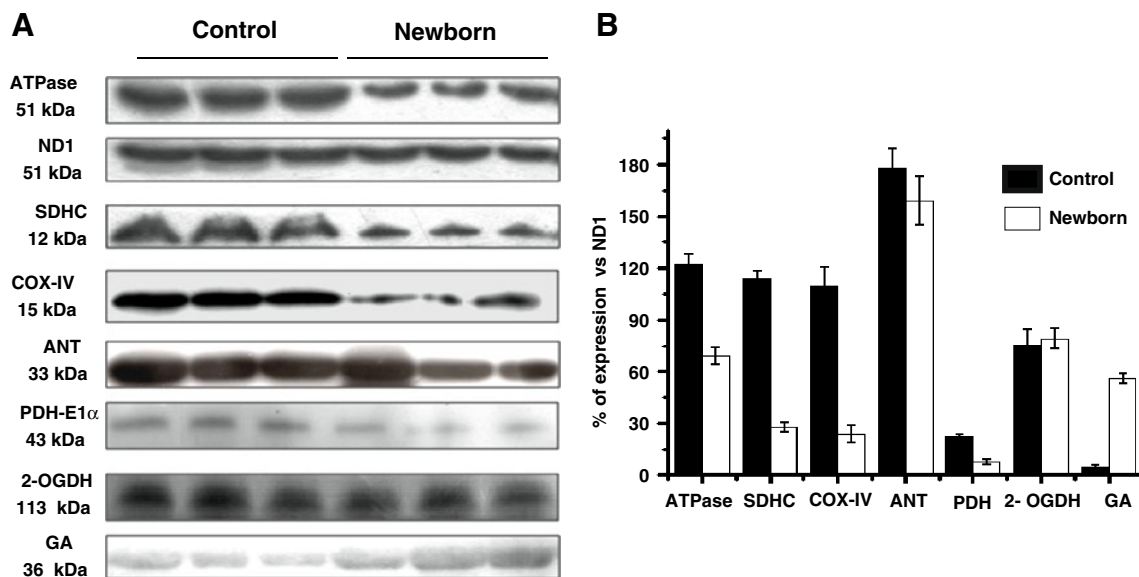
Fig. 8 Western blot analysis of proteins from neonate and adult inner membrane mitochondria. Experimental conditions are described under Material and methods. Data are representative of two independent assays. *ATPase* ATPase 5B; *ND1* NADH Ubiquinone oxidoreductase (Complex I); *SDHC* succinate dehydrogenase B; *COC-IV* Cytochrome oxidase subunit 4; *ANT* adenine nucleotide translocase; *PDH-E1α* Pyruvate dehydrogenase subunit E1α; *2-OGDH* α-ketoglutarate dehydrogenase; *GA* glutaminase

1994; García et al. 2006). Consequently, this finding reinforces the fact that after addition of Ca^{2+}, newborn mitochondria undergo oxidative stress.

Additional important information derived from the electrophoresis study, points out that, in neonate mitochondria, there is an increased expression of some membrane proteins, mainly those that constitute respiratory Complex I, II, and III (Marín-García et al. 1997).

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