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Research Article

Influence of reactive oxygen species production by monoamine oxidase activity on aluminum-induced mitochondrial permeability transition

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Abstract. Treatment of Ca^{2+}-loaded mitochondria with both aluminum and tyramine results in a swelling of higher amplitude than with aluminum alone, while tyramine alone is ineffective. The phenomenon is accompanied by H_2O_2 production and thiol and pyridine nucleotide oxidation. Cyclosporin A, N-ethylmaleimide or dithioerythritol completely prevent these effects, while catalase exhibits a lower inhibition, pointing to the induction of the permeability transition (MPT) by an oxidative stress. Reactive oxygen species are generated by the interaction of aluminum with the inner membrane and the oxidation of tyramine by monoamine oxidase on the outer membrane. This different localization determines the oxidation of critical thiol groups located on both internal and external sides of pore-forming structures, resulting in MPT induction. The reduced effect by aluminum or the inefficacy by tyramine, when implied alone, can be attributable to the oxidation of thiol groups located only on the internal or external side, respectively. Ultrastructural observations show that aluminum plus tyramine induce the typical configuration of mitochondria that have undergone the MPT. Instead, with aluminum alone, the sensitive subpopulation, although swollen, preserves the outer membrane and shows an apparently orthodox configuration.

Key words. Aluminum; mitochondria; permeability transition, monoamine oxidase; tyramine.

Aluminum is a well-known neurotoxic agent whose impact on animal and human health has been demonstrated during the last two decades [e.g. see ref. 1]. Related to this is a large body of literature on the possible involvement of aluminum in Parkinson's and Alzheimer's diseases. However, the claim that aluminum is a contributory etiological cofactor in these diseases is only speculative, and remains to be demonstrated by research currently in progress.

If the effect of aluminum has been extensively studied in neurology, little is known about its toxic effect on other organs and tissues. A well-documented etiological role of aluminum has been reported in certain pathologies such as osteomalacia and microcytic anemia [2]. The molecular mechanisms underlying aluminum toxicity are not yet fully understood, owing to the complexity of aluminum chemistry in aqueous solutions at physiological pH [3–5]. At the subcellular level, as observed in liver, the effect of this element seems to be related to the impairment of the mitochondrial oxidative energy metabolism [6]. Indeed, biochemical and ultrastructural observations demonstrate that aluminum induces in a mitochondrial subpopulation, a phenomenon resembling the membrane permeability transition (MPT) but without membrane potential disruption and Ca^{2+} loss [7].

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The MPT is a phenomenon due to the opening of a high-conductance channel, the transition pore, in the mitochondrial membranes. The phenomenon takes place when a large amount of Ca^{2+} has accumulated in mitochondria, together with an inducing agent, e.g. phosphate or a pro-oxidant agent. The production of reactive oxygen species (ROS) has been suggested to be involved in the opening of the transition pore. The MPT is characterized by collapse of membrane potential, matrix swelling, oxidation and loss of endogenous pyridine nucleotides and release of endogenous cations. All these events lead to a redox catastrophe and a bioenergetic collapse [for a review on the MPT see ref. 8]. Again at the mitochondrial level, aluminum is able to activate monoamine oxidase (MAO) [9, 10], a flavin-containing enzyme located on the outer mitochondrial membrane. This enzyme catalyzes oxidative deamination of biogenic monoamines to their corresponding aldehydes, with H_2O_2 production. Monoamine oxidation has been reported to be responsible for the MPT induction in liver mitochondria provided that phosphate is present [11]. Two isoforms of MAO (MAO-A and MAO-B) have been identified on the basis of their distinct reactivity to various substrates; their cDNAs have been cloned and their deduced amino acid sequences compared [12]. The MAO-B activity is particularly high in rats, a property related to the resistance of this animal species to dopaminergic neurotoxins [13]. In humans, MAO-B activity increases with age and is especially elevated in neurodegenerative diseases [14].

Aluminum has been proposed activate mitochondrial MAO in producing ROS [9, 10]. However, ROS produced by MAO substrates are not able to induce the MPT in the absence of phosphate [11]. The aim of this paper was to assess the effect of aluminum in the presence of an MAO substrate such as tyramine at the level of MPT induction in aluminum-resistant mitochondria. Another aim was to evaluate changes in the ultrastructure of aluminum-sensitive and -resistant mitochondria when exposed to tyramine oxidation.

Materials and methods

Chemicals

Aluminum solutions were prepared as described elsewhere [5]; briefly, a 10 mM aluminum lactate solution prepared in Tris-HCl buffer, pH 7.5, was centrifuged and then passed through a 0.2-µm Millipore microfilter to eliminate all precipitated $Al(OH)_3$. The concentration of aluminum in the resulting clear solution was determined colorimetrically [15]. Dithioerythritol (DTE), EGTA, Hepes, horseradish peroxidase, N-ethylmaleimide (NEM), rotenone, scopoletin and tyramine hydrochloride were purchased from Sigma-Aldrich. Catalase was from BDH. All other reagents were of the highest quality available.

Mitochondria isolation and standard incubation procedures

Rat liver mitochondria (RLM) were isolated by conventional differential centrifugation in a buffer containing 250 mM sucrose, 5 mM Hepes (pH 7.4) and 1 mM EGTA [16]; EGTA was omitted from the final washing solution. Protein content was measured by the biuret method with bovine serum albumin as a standard [17].

Mitochondria (1 mg protein/ml) were incubated in a water-jacketed cell at 20 °C. The standard medium contained 200 mM sucrose, 10 mM Hepes (pH 7.4), 5 mM succinate, 30 µM Ca^{2+} and 1.25 µM rotenone. Variations and/or other additions are given with each experiment.

Determination of mitochondrial functions

Mitochondrial swelling was determined by the change in the absorbance (ΔA) of mitochondrial suspensions at 540 nm using a Kontron Uvikon mod. 922 spectrophotometer equipped with thermostatic control.

The H_2O_2 production was determined fluorometrically by the scopoletin method [18]. Mitochondria at a concentration of 0.5 mg/ml were incubated at 20 °C in standard medium containing 0.5 µM horseradish peroxidase and 1 µM scopoletin. The fluorescence was then evaluated using a Shimadzu RF-5000 spectrofluorophotometer with an excitation wavelength of 350 nm and emission at 460 nm. For a quantitative determination, fluorometric calibration curves were prepared under the same experimental conditions using serial concentrations of commercial H_2O_2.

The protein sulfhydryl oxidation assay was performed as in Bindoli and Rigobello [19].

The oxidation or reduction of pyridine nucleotides in the mitochondrial suspension was followed in an Aminco-Bowman 4-8202 spectrofluorometer operating at excitation and emission wavelengths of 366 and 450 nm, respectively, with a slit of 5 nm.

Electron Microscopy

Samples of 1 ml of mitochondrial suspensions incubated in different conditions were withdrawn after 15 min and then centrifuged for 5 min in an Eppendorf 5A15C at 13,000 g. The pellets were fixed in 2.5% glutaraldehyde, postfixed in 1% OsO_4 in 0.1 M cacodylate buffer, embedded in Epon 812 and then prepared according to standard procedures for transmission electron microscopy (TEM). The image was obtained by a Hitachi H 600.

Results

Energized RLM with a membrane potential value of –180 mV and a respiratory control index of 8 (data not reported), when suspended in standard medium, in the presence of 30 µM Ca^{2+} and treated with 50 µM aluminum

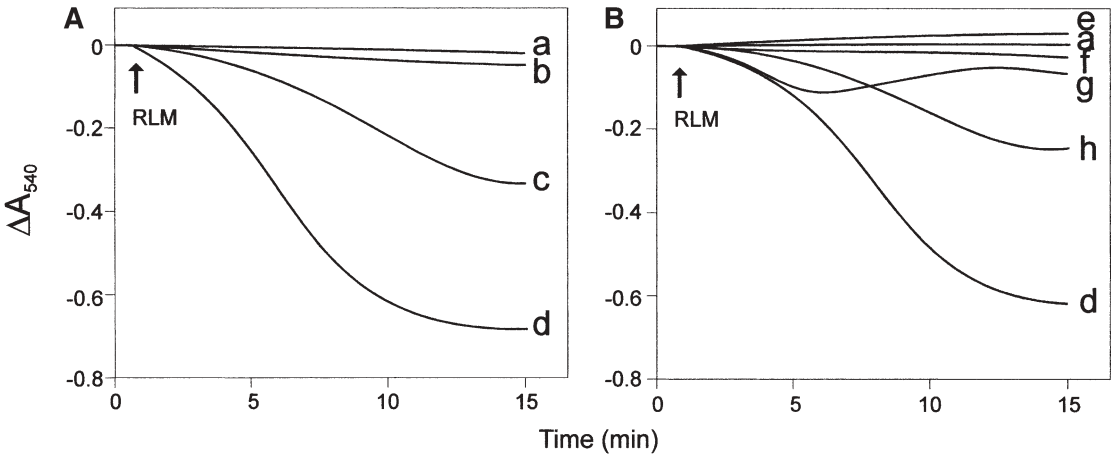

Figure 1. Mitochondrial swelling induced by aluminum plus tyramine. Inhibition by cyclosporin A (CsA), catalase, DTE and NEM. RLM were incubated in standard medium under the conditions indicated in Materials and methods. When present: 50 µM aluminum lactate, 100 µM tyramine, 1 µM CsA, 2 µM catalase, 5 mM DTE and 10 µM NEM. A downward deflection indicates an apparent absorbance decrease. Five additional experiments exhibited the same trend. For all panels, the medium was supplemented with: trace *a*, no further addition; trace *b*, tyramine; trace *c*, aluminum; trace *d*, tyramine and aluminum; trace *e*, tyramine, aluminum and CsA; trace *f*, tyramine, aluminum and NEM; trace *g*, tyramine, aluminum and DTE; trace *h*, tyramine, aluminum and catalase.

lactate exhibit an apparent decrease in absorbance at 540 nm (fig. 1A, trace c) indicative of mitochondrial swelling and MPT induction. The reduced extent in ΔA decrease (about 0.3 units) has been ascribed to the involvement of a subpopulation of mitochondria sensitive to aluminum [see also ref. 7]. No apparent change in absorbance is detectable in the absence of either aluminum or Ca^{2+} (fig. 1A, trace a) or when aluminum lactate is substituted by sodium lactate (not shown).

The presence of 100 µM tyramine, alone, an MAO-A substrate, in the incubation medium (not containing phosphate), does not induce any change in the apparent absorbance of the suspension (fig. 1A, trace b), while addition of both aluminum and tyramine (fig. 1A, trace d) strongly amplifies the swelling compared to aluminum alone (trace c), suggesting a kind of synergistic effect in inducing the MPT. The presence of cyclosporin A (CsA), a specific MPT inhibitor, completely prevents the mitochondrial swelling induced by aluminum plus tyramine (fig. 1B, trace e). The addition of an alkylating or a reducing agent such as NEM or DTE, respectively, inhibits the absorbance decrease (fig. 1B, traces f, g), as well as catalase (fig. 1B, trace h), also if this effect is delayed with DTE and partially with catalase. These observations suggest the involvement of an oxidative stress in the MPT induction in which H_2O_2 and most likely other ROS are involved.

The results reported in figure 2 show the production of H_2O_2 in the mitochondrial suspension visualized as scopoletin oxidation. Aluminum alone induces the production of an apparently low amount of hydrogen peroxide that, however, is able to cause the opening of the transition pore although to a reduced extent (see fig. 1). Tyramine instead, due to the activity of MAO, produces a large amount of H_2O_2 (fig. 2) that, however, is related to a complete lack of MPT induction (fig. 1). The presence of aluminum and tyramine together induces an increase in H_2O_2 production. However, in this condition, RLM undergo larger-amplitude swelling than in the presence of aluminum alone (fig. 1).

The main targets of ROS, in order to induce the MPT, are critical membrane sulfhydryl groups (-SH) and pyridine nucleotides. The histogram in figure 3 shows the changes in the content of reduced thiol groups induced

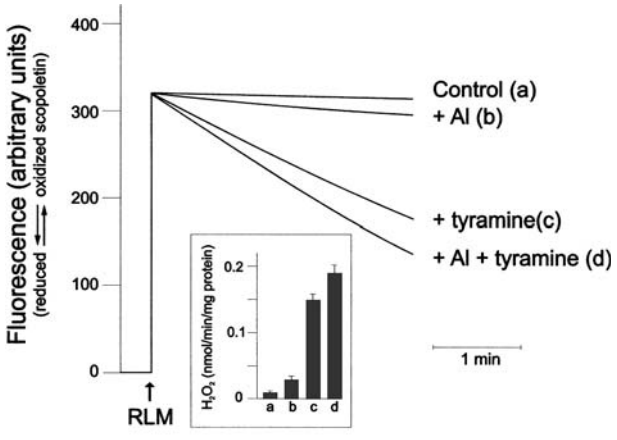

Figure 2. Hydrogen peroxide production induced by aluminium (Al) and tyramine. RLM were incubated as described in Materials and methods; reagent concentrations were as in the legend to figure 1. Reported is a typical experiment of scopoletin oxidation, induced by H_2O_2. Four other experiments gave similar results. The inset reports the initial rates of hydrogen peroxide production (see Materials and methods). Values are means of five observations ± SD. Statistical analysis was carried out using the Student t test (*$p < 0.05$ for all conditions vs control).

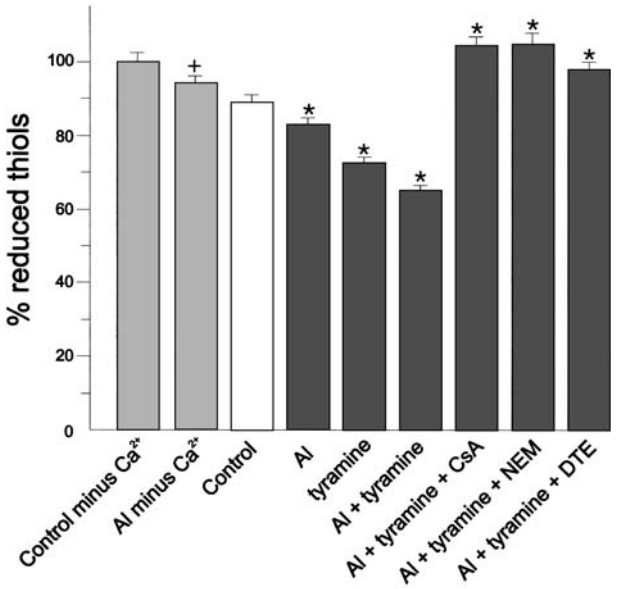

Figure 3. Oxidation of mitochondrial thiols induced by aluminum (Al) and tyramine. Mitochondria were incubated for 15 min under the same experimental conditions and reagent concentrations as in the swelling assays (fig. 1). Where indicated, Ca^{2+} was omitted. Values are means ± SD with different preparations. Statistical analysis was carried out using the Student t test ($^{+}p < 0.05$ vs control minus Ca^{2+}; $^{*}p < 0.05$ vs control), n = 3.

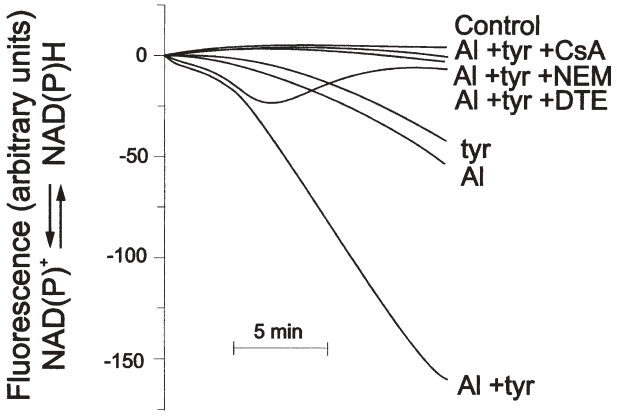

Figure 4. Pyridine nucleotide oxidation by aluminum and tyramine. Effect of CsA. RLM were incubated in standard medium under the conditions indicated in Materials and methods; reagent concentrations were as in the legend to figure 1. When present, 50 µM aluminum lactate (Al), 100 µM tyramine (tyr) and 1 µM CsA. The assay was performed four times with comparable results.

by aluminum and tyramine, alone or together, in the presence of Ca^{2+}. As reported in this figure, where RLM are incubated with Ca^{2+} without any other addition (control), reduced thiol groups decrease by about 10% compared with the condition in the absence of Ca^{2+} (control $-Ca^{2+}$). In the presence of aluminum, –SH groups further decrease by about 18%, while in the presence of tyramine, thiol oxidation is about 28%. Of note is that in the absence of Ca^{2+}, aluminum induces a decrease of reduced thiol groups of 6%. Finally, in the presence of both inducers, the oxidation is still more emphasized, to 35%. The presence of CsA and NEM completely prevent the oxidation and even enhance the content of reduced thiols. DTE also maintains the same level of reduction as the control.

The changes in the redox state of pyridine nucleotides reported in figure 4 follow a similar trend as that observed for thiol oxidation in figure 3, except for the control condition, which does not exhibit any change.

As shown in figure 4, aluminum alone is able to induce a decrease in the fluorescence intensity of the mitochondrial suspension, indicative of pyridine nucleotide oxidation. Tyramine also causes a similar decrease in fluorescence. Aluminum and tyramine, when added together, lead to a much higher decrease in the fluorescence intensity, demonstrating severe pyridine nucleotide oxidation. The presence of CsA and NEM completely prevent this phenomenon, while DTE, as observed on swelling measurements (see fig. 1B), exhibits a delayed effect.

The experiments reported in figure 5 and 6 were performed to evaluate the ultrastructural changes of mitochondria exposed to either tyramine or aluminum or both together, and to visualize the previously proposed presence of two mitochondrial subpopulations, one sensitive and another resistant to aluminum [7]. Figure 5 shows a survey at low magnification of mitochondria in order to observe the general configurations of most of the organelles incubated in the different conditions. Figure 6, instead, shows some typical mitochondria, at high magnification, characterizing each experimental condition. The typical condensed configuration of liver mitochondria incubated in a hypotonic medium with a succinate-supported electron flow and accumulating Ca^{2+} [20] is observable in the micrograph of figure 5A. This mitochondrial configuration, which is representative of the control condition, is clearly observable in the enlargement of figure 6A, and shows roundish RLM in which the matrix spaces have shrunk considerably and voluminous intermembrane spaces are visible. The presence of tyramine does not modify the dimension and the configuration of RLM but clearly induces a strong augmentation of electron-dense areas (figs 5B, 6B). The electron micrograph of mitochondria incubated with aluminum shows that the majority of the organelles exhibit the same dimensions as the control, however several are clearly swollen (fig. 5C). The enlargement in figure 6C demonstrates that the aluminum-resistant mitochondria exhibit, like the control, the condensed configuration, while the swollen ones have a matrix content with an lowerer electron density. This latter type of mitochondria, the so-called aluminum-sensitive subpopulation, seems to exhibit the configuration transition from a condensed to an orthodox form and maintains the outer membrane. The electron micrographs of mitochondria incubated with aluminum and tyramine

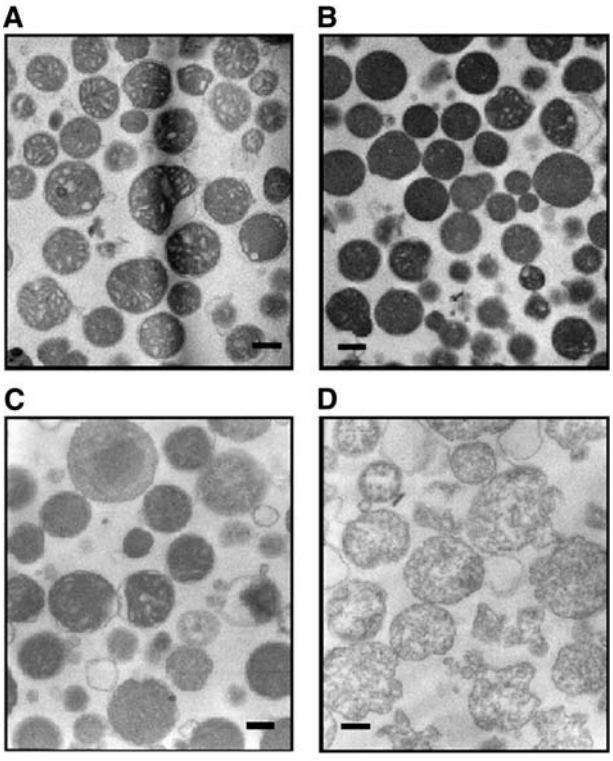

Figure 5. Aluminum-induced mitochondrial swelling. TEM photograph of untreated RLM (*A*), exposed to 100 µM tyramine (*B*), 50 µM aluminum (*C*), 100 µM tyramine + 50 µM aluminum (*D*). Experimental conditions as reported in Materials and methods. Scale bar, 150 nm.

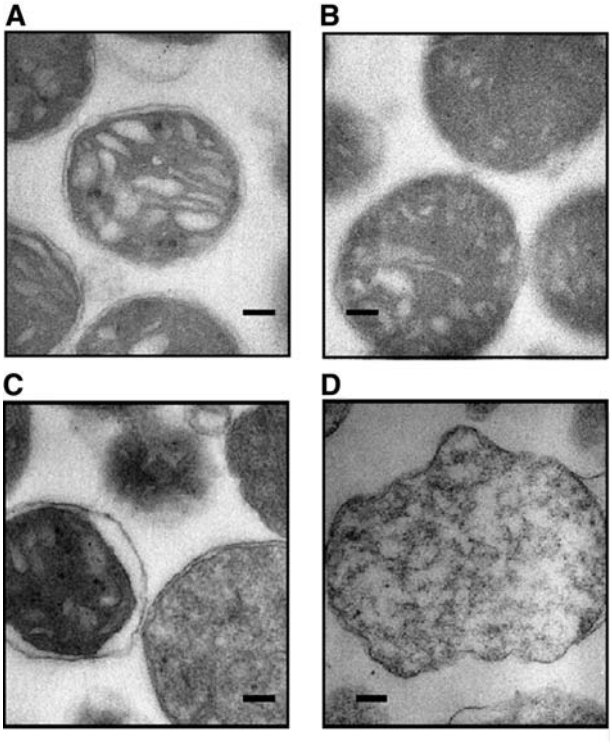

Figure 6. Morphological alterations of RLM induced by aluminum. Experimental conditions and reagent concentrations as in figure 6. Scale bar, 50 nm.

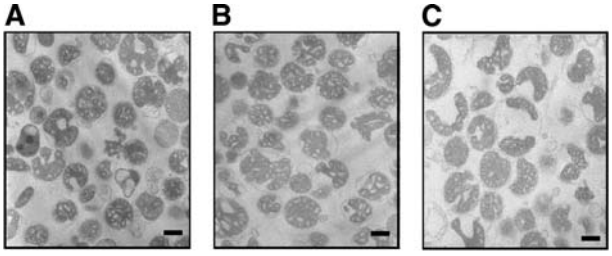

Figure 7. Effects of CsA (*A*), NEM (*B*) and DTE (*C*) on the morphological alterations of RLM induced by aluminum plus tyramine. Experimental conditions and concentrations of aluminum and tyramine as in figure 5. When present: 1 µM CsA, 10 µM NEM, 5 mM DTE. Scale bar, 150 nm.

together show for almost the total population, the typical configuration of mitochondria that have undergone the MPT (fig. 5D). In fact, as shown in figure 6D, they are swollen and 'ghostlike' in appearance, their matrix content having a very low apparent electron density, while the cristae organization has disappeared. Indeed, the RLM have to a large extent lost the outer membrane and assume the characteristic wrinkled profile typical of the increase of the inner compartment and release of Ca^{2+} and other ions [20]. Where the outer membrane is still present, the mitochondrial profile remains roundish. However, RLM exposed to the action of both aluminum and tyramine, and treated with CsA, NEM and DTE (fig.7 A–C, respectively), show again the condensed configuration observed in untreated mitochondria (see figs. 5A and 6A). The same configuration is also observed when RLM are incubated with either aluminum or tyramine without Ca^{2+}, and treated with the above-mentioned inhibitors (result not reported).

All the experiments were performed in a sucrose hypotonic medium in order to compare the results with those obtained in previous investigations on the MTP [7]. However, the use of a KCl isotonic medium has given similar results (not reported). Indeed, the use of β-hydroxybutyrate also gave identical results (not reported).

Discussion

The results reported in this paper show that aluminum, which when incubated alone induces the MPT to a small extent in a mitochondrial subpopulation [7], can cause the same phenomenon, but of large amplitude, in the total mitochondrial population when incubated in the presence of the monoamine tyramine (fig. 1). The mechanism by which aluminum induces the MPT of reduced extent is not completely clarified, even though some proposals have been made. In analogy with observations made on the interactions of aluminum with mitochondrial voltage depentent anion channels (VDACs) [21, 22], it is possi-

ble that nitrogens of a number of closely spaced histidine residues present in AdNT, act in a coordinate manner to chelate the active form of aluminum, that is $Al(OH)_4^-$. The result is an octahedral coordination around the metal that would in turn predispose the membrane to triggering the MPT phenomenon [7].

The observation that the VDAC is a constituent of the transition pore [8], together with other proteins such as AdNT, hexokinase and creatine kinase [23, 24] supports this hypothesis. Indeed, as reported in figure 2 and also previously demonstrated [7], aluminum can provoke the generation of a certain amount of H_2O_2 and most likely other ROS by interacting with the respiratory chain at the level of complex III [7] with the consequent oxidation of critical thiol groups (fig. 3) and pyridine nucleotides (fig. 4) which trigger pore opening to a reduced extent in a mitochondrial subpopulation sensitive to aluminum.

Tyramine alone, despite its capacity to produce a large amount of H_2O_2, much more than aluminum (fig. 2), with consequent thiol and pyridine nucleotide oxidation (figs 3 and 4, respectively), is completely ineffective in triggering pore opening (fig. 1). To explain this result, one needs to take into account that H_2O_2 generation by tyramine has a different orientation compared to that of aluminum, the former being due to the activity of MAO, which is located on the outer membrane, while the latter generation is at the level of the inner membrane [7]. This is confirmed by the observation that exogenous catalase can inhibit H_2O_2 produced by tyramine (fig. 1A) but is ineffective when it is produced by aluminum [7]. The partial effect exhibited by catalase can account for the presence of other ROS derived from H_2O_2.

These results indicate that the thiols oxidized by the products of tyramine catabolism are located on the outer side of the pore-forming structures, while the thiols oxidized by aluminum interaction are located inwardly, and oxidation of the outward -SH groups is not sufficient to trigger pore opening. The observed cooperation between aluminum and tyramine in inducing the MPT in the mitochondrial subpopulation previously resistant to aluminum can thus be attributed to the contemporaneous oxidation of both outer and inner -SH groups. This would exclude a previous proposal in which the observed synergism was suggested as being be due to an activation of MAO by aluminum [9, 10]. On the other hand, the results in figure 2 and 3 clearly show that the generation of H_2O_2 and the consequent oxidation of thiols observable in the presence of tyramine plus aluminum is the sum of the effects obtained by tyramine or aluminum alone, rather than the result of MAO activation.

The proposal of an involvement of critical thiol group oxidation in inducing the MPT by aluminum and the oxidation products of tyramine is in agreement with previous observations indicating that pore opening requires the oxidation of -SH groups [25, 26] located on adenine nucleotide translocase [27]. The oxidation of thiols observed in the presence of Ca^{2+} alone (fig. 3) is most likely ascribable to its interaction with membrane phospholipids leading to membrane disorganization, alteration of coenzyme Q mobility and consequent ROS production [28]. However, the oxidation of these thiols would not participate in MPT induction. The observation that thiol oxidation is paralleled by pyridine nucleotide oxidation in inducing the phenomenon is also in agreement with other studies indicating that two types of site are involved. The first should be an oxidation-reduction-sensitive dithiol, while the second, chemically undefined, is in apparent equilibrium with the pyridine nucleotide pool [29, 30]. The effects of NEM and DTE reported in figures 1B and 4 support the hypothesis of the involvement of internal and external -SH group oxidation in the induction of MPT by aluminum plus tyramine. NEM, which is able to cross the inner membrane, maintains the reduction of both the outer and inner sulfhydryl groups, sensitive to tyramine and aluminum effects, respectively, and completely prevents mitochondrial swelling (fig. 1B) and pyridine nucleotide oxidation (fig. 4). DTE, to which the membrane is impermeable, should be able to reduce only the external thiols, those sensitive to tyramine oxidation. However, this effect is not sufficient to protect mitochondria against the effect of aluminum, which is able to induce the MPT, although of short duration. This permits the internalization of DTE that is then able to reduce the internal -SH groups. This effect is clearly observed by the initial inefficacy of DTE in protecting against mitochondrial swelling (fig. 1B) and pyridine nucleotide oxidation (fig. 4), followed by a subsequent inhibition of both events.

The result reported in figure 3, showing that CsA completely prevents thiol oxidation induced by aluminum plus tyramine in the presence of Ca^{2+}, provides evidence of a particular aspect of this inhibitor in the prevention of MPT induction. In fact, CsA, besides inhibiting -SH oxidation when it is a consequence of pore opening (RLM incubated as mentioned above or with aluminum plus Ca^{2+}), is also able to inhibit this oxidation when the pore remains closed (RLM incubated with Ca^{2+}, or aluminum, or tyramine, alone, or with aluminum plus Ca^{2+}), that is, when thiol oxidation is the cause of subsequent pore opening. This latter effect is explainable taking into account that the first event of MPT induction by the pro-oxidant agents is the oxidation of critical -SH groups located on AdNT [27]. This oxidation enhances the binding of cyclophylin D (Cyp-D) to the translocase, which stabilizes its 'c' configuration, with the result of pore opening in the presence of Ca^{2+} [27]. When CsA is added in these conditions, in which the pore is closed, its interaction with Cyp-D subtracts this protein from binding to AdNT, so stabilizing its 'm' configuration with the result that pore closure is maintained. This stabilization would protect the critical thiols against the oxidation induced by

aluminum and, most likely, by tyramine. The same stabilizing effect can be attributed to the inhibition of -SH oxidation by CsA observed in the presence of Ca^{2+} alone. As mentioned above, Ca^{2+} displaces AdNT from its interaction with membrane cardiolipin and by doing so allows structural changes leading to -SH oxidation [27, 31].

The ultrastructure observations in figures 5C and 6C confirm previous observations that in the presence of aluminum alone, only a subpopulation of mitochondria is sensitive to its effect [7]. However, the present paper also demonstrated that if the resistant mitochondria maintain the condensed configuration of the controls (figs 5A, 6A), sensitive mitochondria exhibit swollen, orthodox configuration form, suggesting a conformational transition from the condensed, induced by aluminum. This configuration would represent the MPT to a reduced extent, confirming previous observations proposing different states of MPT [32]. The micrographs in figures 5B and 6B obtained in the presence of tyramine alone do not show particular changes in the dimensions and configuration of the mitochondria. The observed apparent increase in the electron density of the matrix is under investigation. Finally, when mitochondria are exposed to both aluminum and tyramine they exhibit the typical configuration of organelles that have undergone pore opening. In other words, the different configurations of aluminum-sensitive (orthodox) and aluminum-resistant (condensed) mitochondria, in the presence of tyramine, undergo the transition to the same damaged configuration. The protection exhibited by CsA, NEM and DTE on the MPT induced by aluminum plus tyramine is also observable at the ultrastructural level as these inhibitors are able to prevent the 'ghostlike' swollen configuration, induced by aluminum and tyramine (fig. 7 A–C).

The MPT has been invoked as a mechanism of both apoptotic and necrotic cell death following selected stimuli [33, 34]. Pore formation and apoptosis have also been proposed as a defense system that, by controlling ROS production, can eliminate cells unwanted by the organism [35]. In contrast, the opening of the transition pore and the consequent induction of cellular death in normal cells can also result in a strong toxic effect.

Furthermore, the aluminum-dependent changes in liver mitochondrial membrane permeability here shown with the induction of the MPT, most likely represent the first step in an extensive membrane fusion taking place 'in vivo' by means of a GTP-dependent protein [36–38]. Under the influence of aluminum and ROS, liver mitochondria undergo changes in morphology, i.e. from spherical organelles to a tubovesicular network [33]. This leads to pleomorphic mitochondria and to the formation of megamitochondria typical of liver diseases or cirrhosis [39].

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