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On the mechanisms of phenothiazine-induced mitochondrial permeability transition: Thiol oxidation, strict Ca^{2+} dependence, and cyt *c* release

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ABSTRACT

Phenothiazines (PTZ) are drugs widely used in the treatment of schizophrenia. Trifluoperazine, a piperazinic PTZ derivative, has been described as inhibitor of the mitochondrial permeability transition (MPT). We reported previously the antioxidant activity of thioridazine at relatively low concentrations associated to the inhibition of the MPT (*Brit. J. Pharmacol.*, 2002;136:136–142). In this study, it was investigated the induction of MPT by PTZ derivatives at concentrations higher than 10 μM focusing on the molecular mechanism involved. PTZ promoted a dose–response mitochondrial swelling accompanied by mitochondrial transmembrane potential dissipation and calcium release, being thioridazine the most potent derivative. PTZ-induced MPT was partially inhibited by CsA or Mg^{2+} and completely abolished by the abstraction of calcium. The oxidation of reduced thiol group of mitochondrial membrane proteins by PTZ was upstream the PTP opening and it was not sufficient to promote the opening of PTP that only occurred when calcium was present in the mitochondrial matrix. EPR experiments using DMPO as spin trapping excluded the participation of reactive oxygen species on the PTZ-induced MPT. Since PTZ give rise to cation radicals chemically by the action of peroxidases and cyanide inhibited the PTZ-induced swelling, we propose that PTZ bury in the inner mitochondrial membrane and the chemically generated PTZ cation radicals modify specific thiol groups that in the presence of Ca^{2+} result in MPT associated to cytochrome *c* release. These findings contribute for the understanding of mechanisms of MPT induction and may have implications for the cell death induced by PTZ.

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1. Introduction

The involvement of mitochondrial dysfunction in necrotic or apoptotic cell death is widely described [1,2]. Under several conditions, mitochondria can undergo a sudden permeability transition that is mediated by the opening of the permeability transition pore (PTP) [3,4]. This process, known as mitochondrial permeability transition (MPT), is described as a Ca^{2+}-dependent CsA-sensitive process [5] caused by the opening of a non-specific pore formed between the mitochondrial membranes that makes them permeable to small solutes (<1.5 kDa). MPT can be triggered by Ca^{2+} overload [6], Ca^{2+} plus Pi or prooxidants [7] and protein thiol reactants [8,9] and is accompanied by a large amount of mitochondrial matrix swelling, dissipation of the mitochondrial transmembrane potential, an increase in basal mitochondrial oxygen consumption, mitochondrial calcium efflux and the release of pro-apoptotic proteins such as cytochrome *c* [10]. The induction or inhibition of MPT by several drugs has been extensively investigated in attempts to understand observed side effects or explain novel observed beneficial effects of these drugs, as in the case of the tetracycline derivative minocycline [11].

Phenothiazines (PTZ) are drugs widely used in the treatment of schizophrenia, psychosis and anxiety [12]. For a long time, PTZ derivatives were also used efficiently as anti-emetic drugs [13,14], even in the treatment of chemotherapy-induced emesis [15]. Depending on the substituents, a PTZ can be classified as aliphatic, piperazinic or piperidine derivatives [16]. The more common side effects exhibited by patients who chronically use PTZ are extrapiramidal disturbances [17], *torsades de pointe* arrhythmias and increased risk of sudden cardiac death [18–20], agranulocyto-

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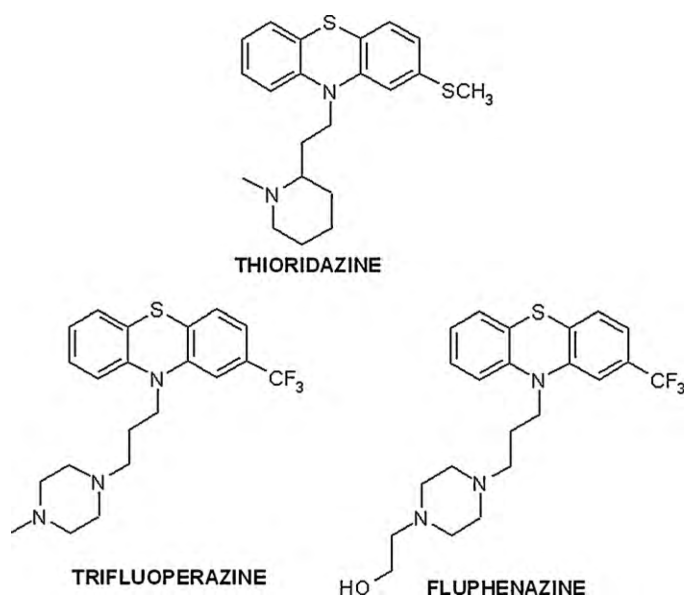


Chart 1. Molecular structure of phenothiazine derivatives thioridazine, trifluoperazine and fluphenazine.

sis [21], and photoallergic eruptions [22,23]. It has also been reported that some patients presented severe hepatotoxicity associated to the clinical use of phenothiazines that includes acute intrahepatic cholestasis [24–26], steatosis [27], and hepatitis [28]. However, the molecular mechanisms of phenothiazine-induced hepatotoxicity are poorly understood.

In addition to the pharmacological and toxicological effects deriving from their clinical prescription, PTZ have been the focus of several biological and physical-chemical investigations. Numerous studies have described trifluoperazine [29] and CsA [30] as MPT inhibitors. Also, the amphiphilic character of PTZ has led to a variety of studies concerning the interaction of phenothiazines with biological membranes (reviewed in [31]). Recently, PTZ-induced cell death was reported in cultured cell lines [32,33]. Although the molecular mechanisms remain unclear, PTZ-induced cell death was associated to the dissipation of mitochondrial transmembrane potential [34], suggesting the involvement of mitochondrial dysfunction in the cytotoxicity observed in activated human lymphoblasts.

Considering that PTZ are able to induce cell death *in vitro* and that MPT plays a central role in this process, this study evaluated the effects of the phenothiazine derivatives thioridazine (TR), trifluoperazine (TFP) and fluphenazine (FP) (Chart 1) as MPT inducers in isolated rat liver mitochondria. The results elucidated the molecular mechanisms of the induction of MTP by PTZ and contribute to a better comprehension of the mechanisms of cell toxicity presented by these drugs.

2. Methods

2.1. Materials

Thioridazine, trifluoperazine, fluphenazine, sucrose, EGTA, HEPES, K_2HPO_4, succinate, rotenone, malonate, oligomycin, ruthenium red, rodhamine 123, 5,5′-dithiobis(2-nitrobenzoic acid, DTBN), o-phthalaldehyde, arsenazo III and $CaCl_2$ were purchased from the Sigma–Aldrich Company (St. Louis, MO, USA). DMPO (2,2′-dimethyl-pyrroline-N-oxide, also from Sigma–Aldrich Co.) was previously vacuum-distilled and purified as recommended elsewhere [35]. KCl, KOH, and KCN were purchased from Merck (Germany). All other reagents were acquired as commercial products of the highest purity grade available and used without further purification. Aqueous solutions were prepared with double distilled-deionized water (mixed bed ion exchanger, Millipore, MA, USA).

2.2. Isolation of rat liver mitochondria (RLM)

Rat liver mitochondria were isolated using conventional differential centrifugation [36]. Donors were Wistar rats weighting approximately 180 g and receiving food and water ad libitum in a light-controlled room (12 h light/dark cycles). Each liver was reached through a bilateral abdominal incision. The organ was sliced and homogenized in 250 mM sucrose, 1 mM EGTA, and 10 mM HEPES-KOH buffer (pH 7.2). Then the mitochondrial suspension was washed twice in the same medium but containing 0.3 mM EGTA. The final pellet was suspended in 250 mM sucrose and 10 mM HEPES-KOH buffer (pH 7.2) to a final protein concentration of 80–100 mg/mL. The isolation procedure was performed at 4 °C. During experiments the mitochondrial suspension was kept on ice. The mitochondrial protein amount was determined using the Biuret reaction [37]. All studies using isolated mitochondria were performed within a 3-h period.

2.3. Mitochondrial swelling

RLM (0.27 mg protein/mL) were added to a buffer containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.4, at 30 °C plus 5 mM potassium succinate, 2.5 μM rotenone and 10 μM $CaCl_2$ added in the presence or absence of phenothiazines and PTP modulators. Mitochondrial swelling was estimated from the decrease in relative absorbance at 540 nm in function of time using a Hitachi U-2000 Spectrophotometer (Tokyo, Japan). In the presence of PTP modulators mitochondrial swelling was evaluated by initial-end point measurements of relative absorbance or turbidity at 540 nm (ΔA_{540nm}). Energized mitochondria were pre-incubated with each modulator for 30 s before the addition of TR and the turbidity was measured immediately and then after 10 min. Other experimental details are described in the figure captions.

2.4. Thiol quantification

Mitochondrial membrane proteins were selected by the freeze-thawing method [38] and reduced thiol groups were quantified using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). After 10 min incubation under swelling conditions, the mitochondrial suspension was submitted to three subsequent freeze-thawing cycles to release matrix proteins, and then centrifuged for 15 min at 6000 × *g*. The pellet was treated with 0.2 mL of 6% trichloroacetic acid and centrifuged at 6000 × *g* for 15 min to precipitate the mitochondrial membrane proteins. The final pellet was suspended with 1 mL of 0.5 M potassium phosphate buffer, pH 7.6, containing 0.4% SDS. After the addition of 0.1 mM DTNB, absorbance was determined at 412 nm and the amount of reduced thiol groups was calculated from $\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$ [39].

2.5. SDS-polyacrylamide slab gel electrophoresis of mitochondrial proteins

The same samples obtained in the protein thiol content assay by freeze-thawing procedures were submitted to the electrophoresis. Samples were boiled for 2 min in 250 mM Tris–HCl, pH 7.4, 20% SDS, and 50 mM EDTA according to Liu et al. [40]. An aliquot (0.4 μg) was applied to the electrophoresis gel and the SDS-PAGE was performed in a discontinuous system as described by Laemmli [41]. After separation at voltage was 20 mA, the gel (3.5% stacking gel and 8% running gel in acrylamide) was stained with silver nitrate as described by Blum et al. [42].

2.6. Estimation of GSH levels

After a 10 min incubation under swelling conditions, 0.2 mL of 6% trichloroacetic acid was added to the mitochondrial suspension followed by centrifugation at $6000 \times g$ for 15 min. Supernatant was taken to the measurement of mitochondrial reduced gluthathione content by a fluorimetric assay under experimental swelling conditions employing ortho-phthalaldehyde (OPT) in a Hitachi F-2500 spectrofluorometer (Tokyo, Japan) using 350/420 nm excitation/emission wavelengths [43].

2.7. Spin trap EPR measurements

Electron Paramagnetic Resonance (EPR) spectra were recorded at room temperature (22 ± 2 °C) on a Bruker EMX EPR spectrometer (Bruker Optics Inc., Billerica, MA, USA) equipped with a standard cavity, operating at X-band frequency, using a standard flat quartz cell. Instrument conditions were usually 1.00×105 gain, 1.5 G modulation amplitude with a resolution of 1024 points. The magnetic field was calibrated with 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPOL, $g = 2.0056$) [44,45]. The EPR spin trapping experiments were performed in the swelling assay medium previously treated with Chelex-100 resin to remove contaminant metal ions. A stock solution of 0.1 M DBNBS was prepared in phosphate buffer pH 7.4. In a typical spin trapping experiment, the spin trap solution (10 mM DBNBS or 80 mM DMPO) was added to the mitochondrial suspension in sucrose buffer in a polyethylene vial, and 0.2 mL was immediately transferred to a flat quartz cell. The EPR spectra were registered no later than 1 min after transfer. The spectra were registered at different time intervals to monitor the EPR signal evolution. Appropriate controls were performed.

2.8. Mitochondrial transmembrane electrical potential ($\Delta\Psi$)

Mitochondrial $\Delta\Psi$ was estimated by noting changes in the rodhamine 123 fluorescence (0.4 μM), recorded on a Hitachi F-2500 spectrofluorometer (Tokyo, Japan) operating at 505/525 nm excitation/emission wavelengths, respectively, with a excitation/emission slit width of 5 nm. The results were expressed as percentages of dissipation in relation to uncoupled mitochondria (1.0 μM CCCP).

2.9. Mitochondrial Ca^{2+} efflux

Calcium retention in isolated mitochondria was estimated by noting the difference between the absorbance spectra of arsenazo III at the wavelength pair 675 and 685 nm [46], in a photodiode array spectrophotometer (Shimadzu Multispec 1501, Tokyo, Japan). RLM (0.27 mg protein/mL) was added in a buffer containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.4, at 30 °C plus 2.5 μM rotenone 10 μM $CaCl_2$, and 10 mM arsenazo III. Mitochondrial Ca^{2+} uptake was triggered by adding 5 mM potassium succinate. After reaching the steady-state, PTZ was added to the reaction.

2.10. Recombinant EP24.15 expression and incubation with TR

Recombinant EP24.15 (Thimet oligopeptidase; TOP; MW 78 kDa) was expressed in *E. coli* as a glutathione S-transferase (GST) fusion protein using the expression vector pGEX-4T2 (Amersham Biosciences, England). Protein purification was initially done by affinity chromatography using a glutathione Sepharose 4B column (Amersham Biosciences) with the protein released from the GST fusion by cleavage with thrombin (0.5 U; Amersham Biosciences). The purity of the protein was analyzed. After confirming that the EP24.15 homogeneity was larger than 95% by Coomassie brilliant blue staining after 12% SDS-PAGE (not shown), the protein was stored at −80 °C in small aliquots.

For partial reduction of its thiol groups, EP24.15 was incubated with 1 mM Tris(2-carboxyethyl) phosphine (TCEP), a specific thiol reductant, followed by filtration through PD-10 (GE) to completely remove the reductants, thus reaching approximately 40% reduction of the thiol groups. For the experiments, 1.5 μM EP24.15 was incubated with 4.5 μM TR for 15 min at 25 °C in the dark and irradiated. The irradiation was performed using a 4-W lamp (Entela Mineralight Lamp Multiband UV Model UVGL-25) at 254 nm.

2.11. Determination of the reduced cysteine residues of EP24.15

The assay was performed as described previously [47]. Briefly, 0.25 mg of sulfhydryl-modified EP24.15 was resuspended in 0.3 mL of 30 mM Tris, pH 7.4, containing 1 mM EDTA and 8 M urea. After complete dissolution, samples were taken for absorbance reading at 280 nm. Afterward, 0.1 mM DTNB (final concentration) was added to the samples and incubated for 40 min in the dark followed by readings at 412 nm. The protein concentration was calculated using the theoretical $\varepsilon_{280} = 78{,}240\ M^{-1}\ cm^{-1}$ for EP24.15. The concentration of the CyS–TNB complex was deduced from $\varepsilon_{412} = 14{,}150\ M^{-1}\ cm^{-1}$. The number of reduced Cys residues was then calculated from the ratio between molar concentrations of the protein and the CyS–TNB complex.

2.12. SDS-polyacrylamide slab gel electrophoresis of EP24.15

The same sample obtained in the protein thiol content assay was used for electrophoresis. Samples were applied to the electrophoresis gel under 20 mA voltage in a SDS-PAGE (3% stacking gel and 8% running gel in acrylamide) and were stained with silver nitrate as described elsewhere [42].

2.13. Quantification of the release of cytochrome c

Cytochrome *c* released from isolated mitochondria was detected through of enzyme immunoassay (Biosource Int, Inc., CA, USA). Mitochondria (0.27 mg protein/mL) were incubated with TR under swelling assay conditions for 10 min and then centrifuged at $16{,}000 \times g$ for 5 min. An aliquot portion of the obtained supernatant (0.05 mL) was added to wells and incubated with a biotin-conjugated monoclonal anti-cyt *c* for 2 h at 25 °C. After five washes, a streptavidin-HRP solution was added and the plate was incubated for 30 min. The reaction was stopped and optical density was determined at 450 nm using a microplate reader (BioTek ELX800, BioTek Instruments, Inc., Vermont, USA). Sample concentrations were quantified based on a standard curve within a 0.08–2.5 ng/mL concentration range ($\varepsilon = 0.595\ ng^{-1}\ mL$).

2.14. Data analyses

Graphical and statistical analyses were performed using Microcal (TM) Origin® version 6.0 (Microcal Software Inc., Northampton, MA, USA) and GraphPad Prism version 3.00 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Induction of mitochondrial permeability transition (MPT) by phenothiazines (PTZ)

The literature labels PTZ as MPT inhibitors (at approximately 10 μM). However, the current study evaluated the effects of higher concentrations of three PTZ derivatives (thioridazine, TR;

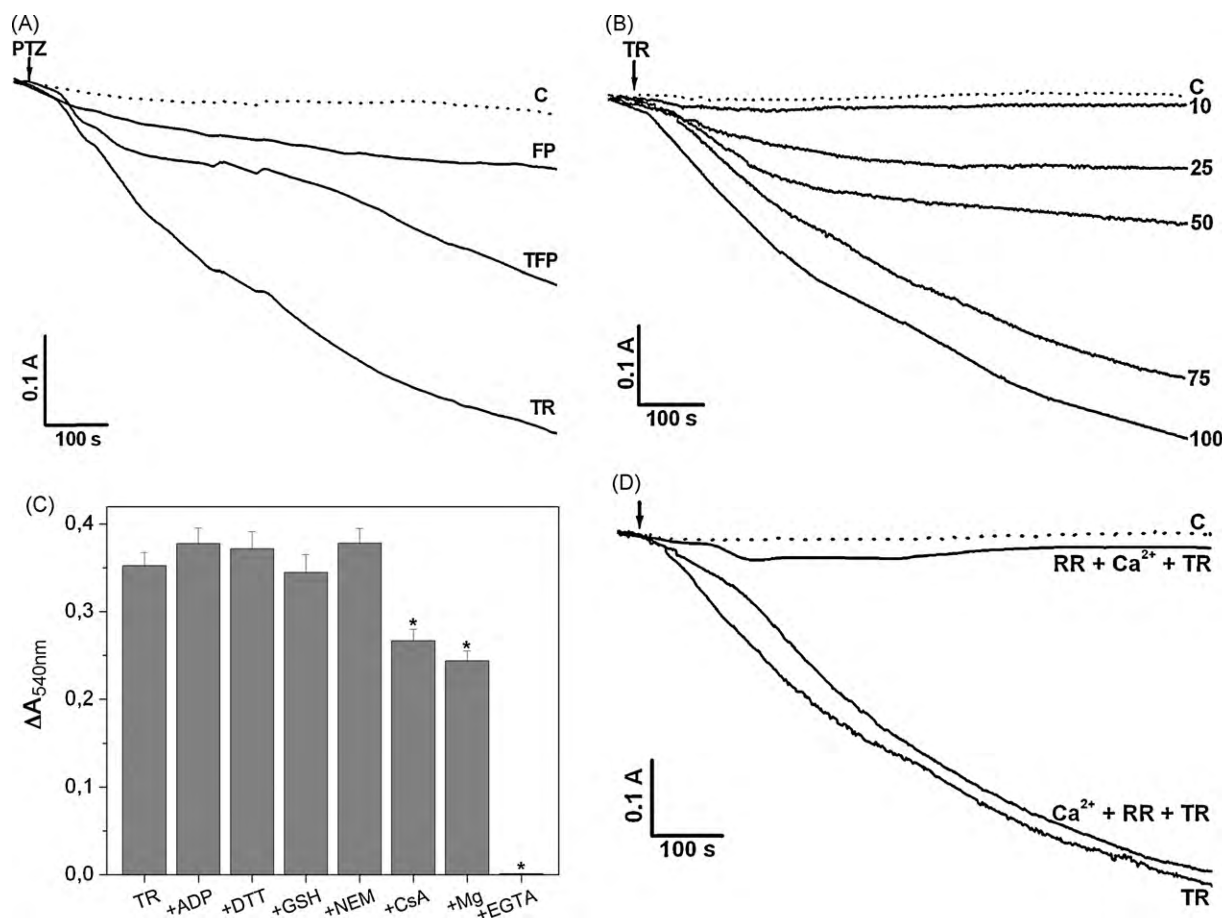


Fig. 1. Mitochondrial swelling induced by phenothiazines. Mitochondria (0.27 mg protein/mL) were incubated in a reaction buffer containing 125 mM sucrose, 65 mM KCl and 10 mM HEPES-KOH, pH 7.4, in the presence of 5 mM succinate, 2.5 μM rotenone, and 10 μM $CaCl_2$, at 30 °C. (A) Swelling was initiated by the addition of 100 μM PTZ indicated by the arrow. Lines: C, control in absence of PTZ; TR, thioridazine; TFP, trifluoperazine; FP, fluphenazine. (B) Effect of the variation of the thioridazine (TR) concentration on mitochondrial swelling; the addition of TR is indicated by the arrow and numbers are concentrations in μM. (C) Effects of MPT modulators on TR-induced mitochondrial swelling Values were presented as mean ± s.d. of three different measurements. Bars: TR, 100 μM thioridazine; +ADP, 0.1 mM adenosine phosphate plus 100 μM TR; +DTT, 0.1 mM dithiothreitol plus 100 μM TR; +GSH, 0.1 mM reduced gluthathione plus 100 μM TR; +NEM, 0.1 mM N-ethylmaleimide plus 100 μM TR; +CsA, 1.0 μM cyclosporine A plus 100 μM TR; +Mg, 0.1 mM $MgCl_2$; +EGTA, 0.1 mM plus 100 μM TR. (*) Statistically different from TR ($p < 0.005$). (D) Effect of ruthenium red (RR) on TR-induced mitochondrial swelling. Lines: C, control, in the absence of RR and TR; RR + Ca^{2+} + TR, 0.5 mM RR was pre-incubated with non-energized mitochondria followed by addition of succinate, rotenone, and calcium and after start recording 100 μM TR was added as indicated by the arrow; Ca^{2+} + RR + TR, mitochondria was added to the reaction buffer containing succinate, rotenone and calcium and then 0.5 mM RR was added; after start recording 100 μM TR was added (arrow); TR, 100 μM TR. In (A), (B), and (D) traces are representative of at least three experiments with different mitochondrial preparations.

trifluoperazine, TFP; and fluphenazine, FP) on the permeability of isolated rat liver mitochondria (RLM). As observed in Fig. 1, adding 100 μM PTZ to the mitochondrial suspension promoted a sudden decrease in the relative turbidity at 540 nm due to the matrix swelling of isolated RLM previously energized with 5 mM potassium succinate (plus 2.0 μM rotenone) in the presence of added 10 μM $CaCl_2$. The addition of PTZ before Ca^{2+} resulted in the same swelling extension exhibited in Fig. 1. TR, the piperidine derivative, promoted the largest swelling extension, followed by the piperazine derivatives TFP and FP, respectively (Fig. 1A). Swelling that indicated nonselective permeabilization of the inner mitochondrial membrane for diffusion of solutes was dependent on the concentration of PTZ. According to previously observed phenomena [48], at 10 μM TR was not able to promote mitochondrial swelling. However, higher concentrations (25, 50, 75, and 100 μM) proportionally increased the observed swelling extension in RLM (Fig. 1B). The same results were obtained using TFP and FP (not shown). Only data on TR is presented because it was the most potent PTZ derivative.

To characterize PTZ-induced mitochondrial permeabilization, the effects of TR on mitochondrial swelling were probed in the presence of different well-known MPT modulators [49]. As shown in Fig. 1A and B, turbidimetric changes in the mitochondrial suspensions were evaluated kinetically in the presence of MPT modulators. Surprisingly, pre-incubation of RLM with EGTA increased the extension of TR-induced swelling. Since the photochemical behavior of PTZ in homogeneous media had previously been characterized [50], experimental controls were applied, revealing that PTZ reacted with EGTA when photoexcited by spectrophotometer lamps (not shown). Thus, instead of kinetics, initial-end point measurements were performed in the dark to avoid such interference. These data were presented as variations in absorbance at 540 nm (Fig. 1C). In this experiment, without the addition of modulators, PTZ exhibited the same swelling extension recorded in Fig. 1A. ADP, which stabilizes the "m" conformation of the adenine nucleotides translocator (ANT), did not inhibit PTZ-induced mitochondrial swelling. Since it is well established that protein thiol oxidation is involved with the MPT trigger [51], we evaluated the effects of DTT, a thiol reducer; NEM, a monothiol hydrophobic ligand; and GSH, a hydrophilic thiol tripeptide that could be oxidized instead of membrane thiols. None of the thiol reactants used was able to prevent TR-induced mitochondrial swelling. On the other hand, cyclosporine A (CsA), the classic MPT inhibitor (due to the inhibition of cyclophilin D

(CypD) binding to the protein complex that composes the pore), partially inhibited the mitochondrial permeabilization induced by 100 μM PTZ. $MgCl_2$, another MPT inhibitor that antagonizes Ca^{2+} binding to a PTP complex, exhibited a similar effect as that observed for CsA, indicating the dependence of this cation. The same pattern of response was obtained for TFP and FP (not shown). EGTA, a specific Ca^{2+} chelator, completely inhibited mitochondrial swelling induced by TR, demonstrating that the PTZ-induced MPT is dependent of Ca^{2+}.

To investigate the role of Ca^{2+} in the PTZ-induced MPT, the uniporter inhibitor ruthenium red (RR) was used in the swelling assays. Before adding respiratory substrate succinate, the mitochondrial suspension was pre-incubated with RR to prevent the mitochondrial Ca^{2+} uptake associated with the formation of the electrochemical gradient. In this condition, the addition of 100 μM TR to the mitochondrial suspension was not able to promote mitochondrial swelling, even when Ca^{2+} was added after RR (Fig. 1D). However, when Ca^{2+} was added to succinate-energized RLM before RR, TR promoted a large amount of mitochondrial swelling. Such findings demonstrate that it is necessary to have Ca^{2+} inside the mitochondria (matrix) for TR to be able to induce MPT.

3.2. Mitochondrial transmembrane potential ($\Delta\Psi$) dissipation and Ca^{2+} efflux related to PTZ-induced MPT

The MPT process is accompanied by dissipation of the electrochemical proton gradient, release of calcium from the mitochondrial matrix, ATP depletion, an increase of ROS generation and activation of cytosolic proteases and endonucleases resulting in cell death [52]. In order to characterize the events associated to PTZ-induced MPT, $\Delta\Psi$ and calcium efflux were measured. As expected, the addition of 100 μM TR, TFP, and FP to succinate-energized isolated RLM promoted an immediate increase of the rodhamine 123 fluorescence. This was due to its release from the mitochondrial matrix in response to the dissipation of mitochondrial transmembrane potential, assayed during experimental conditions of swelling experiments—i.e., in the presence of added calcium (absence of EGTA) (Fig. 2A). Also, the extension of PTZ-induced ($\Delta\Psi$) dissipation took the same order as

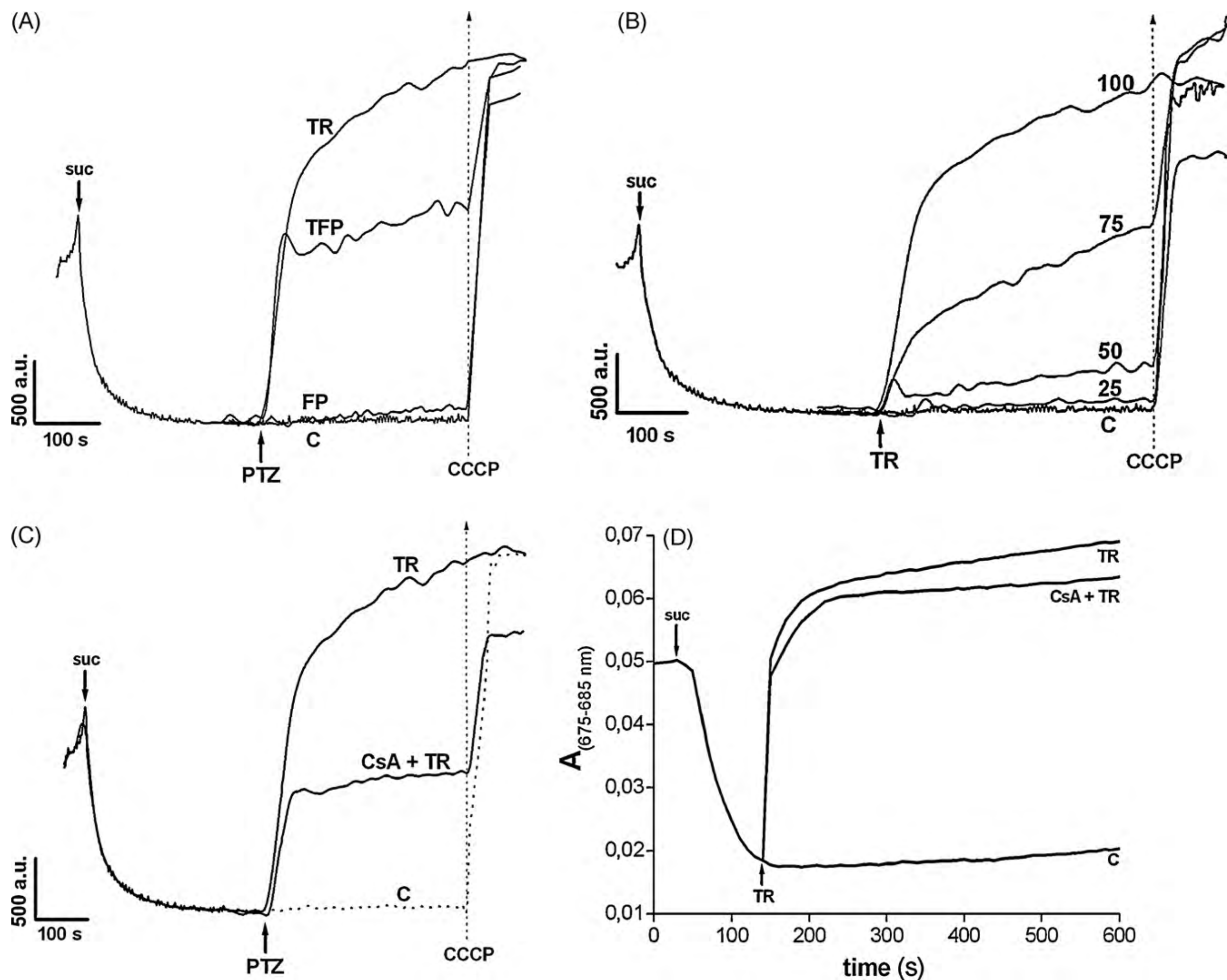


Fig. 2. Dissipation of the mitochondrial transmembrane potential ($\Delta\Psi$) and calcium efflux induced by phenothiazines. Mitochondria were incubated in the experimental conditions described in the captions of Fig. 1. (A) Mitochondria were energized by the addition of 5 mM succinate (suc) followed by addition of PTZ, as indicated by the arrows. Lines: C, control in the absence of PTZ; FP, fluphenazine; TFP, trifluoperazine; TR, thioridazine. (B) Effect of variation of the TR concentration on mitochondrial $\Delta\Psi$; the addition of TR is indicated by the arrow and numbers are concentrations in μM. (C) Effect of cyclosporine A (CsA) on TR-induced $\Delta\Psi$ dissipation. Lines: C, control in the absence of PTZ; TR, 100 μM thioridazine; CsA + TR, pre-incubation of mitochondria with 1.0 μM CsA and addition of 100 μM TR after energization. In (A), (B) and (C) 1.0 μM CCCP was added in the end of all experiments to promote complete $\Delta\Psi$ dissipation (dotted arrow). (D) Ca^{2+} uptake by mitochondria was triggered by energization with succinate (suc) and TR was added after reach the steady-state (arrow). Lines: C, control in the absence of TR; TR, 100 μM thioridazine; CsA + TR, pre-incubation of mitochondria with 1.0 μM CsA and addition of 100 μM TR after Ca^{2+} uptake. Mitochondria were pre-incubated with CsA for 30 s before the addition of TR. Traces are representative of three experiments with different mitochondrial preparations.

that observed for the swelling experiments. Thus, relative to the uncoupler CCCP, TR promoted total $\Delta\Psi$ dissipation, followed by TFP and FP, respectively. As shown in Fig. 2B, such dissipation was responsive to variation in TR concentrations, exhibiting a close correlation with the mitochondrial swelling induced by TR. CsA partially prevented the TR-induced $\Delta\Psi$ dissipation (Fig. 2C), indicating a direct cause–consequence relationship with the opening of the PTP.

The MPT triggered by TR was also accompanied by calcium efflux in isolated RLM. As shown in Fig. 2D, after mitochondrial Ca^{2+} uptake promoted by mitochondrial energization with potassium succinate, the addition of 100 μM TR (after the Ca^{2+} gradient has reached the steady-state) promoted an immediate mitochondrial Ca^{2+} efflux. TR-induced Ca^{2+} release may occur by different mechanisms, including uncoupling or the PTP opening. Since CsA had a partial inhibitory effect under such TR-induced-efflux, similar to the partial inhibition of the TR-induced MPT, we proposed that TR-induced Ca^{2+} release occurs via PTP. Nonetheless, uncoupling was observed at this PTZ concentration [46], however the measurement was performed in the respiration condition, i.e., in the presence of EGTA—a condition that PTZ-induced mitochondrial swelling was not observed in the present study. TFP and FP also promoted mitochondrial calcium efflux with a similar extension of swelling assays (not shown).

3.3. Reactivity of PTZ with mitochondrial thiols

The occurrence of MPT is associated with the cross-linkage of mitochondrial membrane-associated protein thiol groups, resulting in disulfide bond formation [53]. Such oxidation may be caused by the oxidant action of free radicals [54] or by direct action of thiol reagents [55]. For this study, mitochondrial suspensions were incubated with 100 μM PTZ under the same experimental conditions as the investigation of swelling assays and the mitochondrial membrane proteins (isolated from the soluble proteins by the freeze-thawing technique, Fig. 3A) or total mitochondrial proteins (Fig. 3C) were used to thiol determination. After acid precipitation, the number of reduced thiol groups was determined using Ellman's reagent DTNB. As shown in Fig. 3A, PTZ promoted a significant decrease in the reduced mitochondrial membrane protein thiol groups after a 10-min incubation. The prooxidant t-butylhydroperoxide (t-BOOH), which induces mitochondrial swelling associated with the cross-linkage of thiol groups in the presence of Ca^{2+} [56], was used as a positive control in this experiment. It is not possible correlate directly the percentage of swelling extension with the amount of thiol oxidation because swelling is only a turbidimetric estimative and probably other thiol groups not involved with PTP are oxidized by PTZ. However, whether the order was compared, the extent of the PTZ-induced decrease of reduced thiol content was

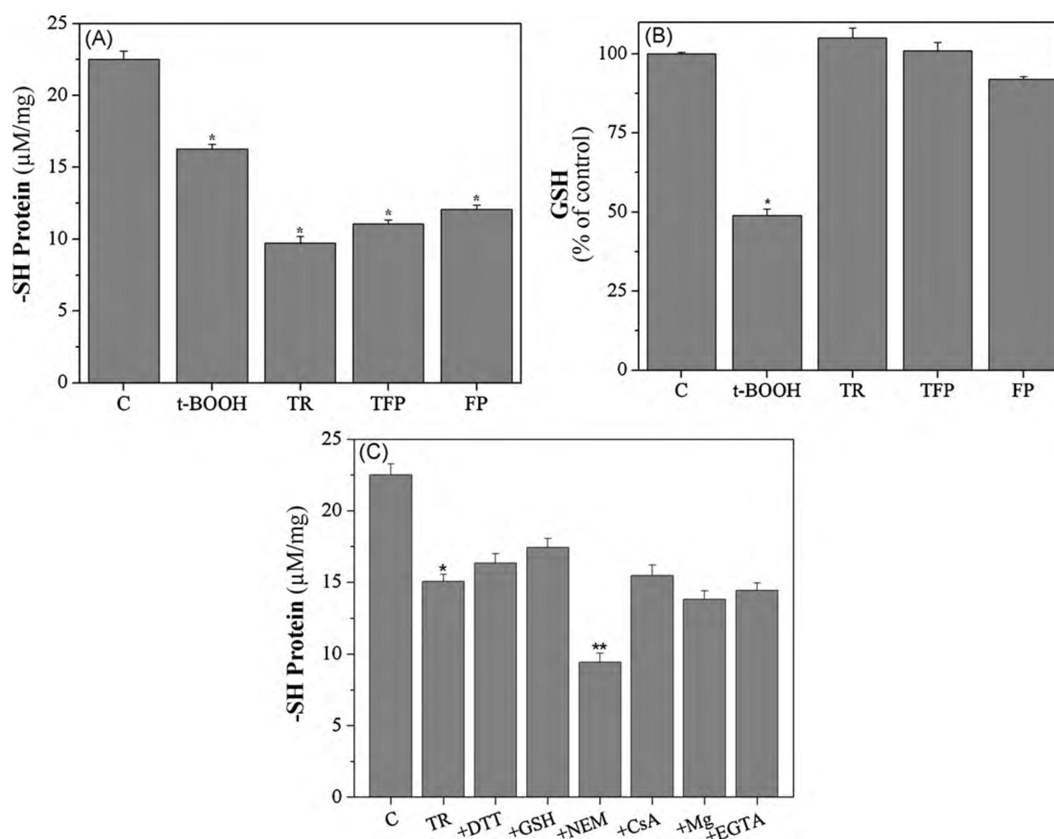


Fig. 3. Effects of phenothiazines on thiol groups of mitochondrial proteins and gluthathione. Mitochondria were incubated in the same experimental conditions of the mitochondrial swelling assay described in Fig. 1 for 10 min and after protein thiol groups and reduced gluthathione (GSH) was measured. (A) Mitochondrial membrane proteins were separated by the freeze-thawing technique and the quantification was performed using DTNB (see Section 2 for details). Bars: C, control in the absence of PTZ; t-BOOH, 0.6 mM *tert*-butyl hydroperoxide as positive control; TR, 100 μM thioridazine; TFP, 100 μM trifluoperazine; FP, 100 μM fluphenazine. (*) Statistically different from C ($p < 0.005$). (B) After acid precipitation of mitochondrial proteins, GSH levels were measured in the supernatant using *o*-phtalaldehyde. Bars: C, control in the absence of PTZ; t-BOOH, 0.6 mM *tert*-butyl hydroperoxide as positive control; TR, 100 μM thioridazine; TFP, 100 μM trifluoperazine; FP, 100 μM fluphenazine. (*) Statistically different from C ($p < 0.005$). (C) Effects of MPT modulators on the oxidation of mitochondrial protein thiol groups by TR; mitochondria were pre-incubated with each modulator for 30 s followed by incubation with TR for 10 min and the total protein thiol groups were quantified using DTNB, as in (A). Bars: C, control in the absence of TR; TR, 100 μM thioridazine; +DTT, 0.1 mM dithiothreitol plus 100 μM TR; +GSH, 0.1 mM reduced gluthathione plus 100 μM TR; +NEM, 0.1 mM N-ethylmaleimide plus 100 μM TR; +CsA, 1.0 μM cyclosporine A plus 100 μM TR; +Mg, 0.1 mM $MgCl_2$; +EGTA, 0.1 mM plus 100 μM TR. (*) Statistically different from C ($p < 0.005$) and (**) statistically different from TR ($p < 0.005$). In (A), (B), and (C) values were presented as mean ± s.d. of three different measurements.

correlated with the ability of PTZ to induce mitochondrial swelling, where TR shows the highest potency followed by TFP and FP, respectively. The oxidant t-BOOH induced a mitochondrial swelling approximately 40% lower than that induced by TR (not shown). It is interesting to note that the thiol oxidation induced by TR was higher than that induced by t-BOOH, although the degree of –SH oxidation was not accompanied by the detection of large amounts of high molecular protein aggregates in electrophoresis (Supplementary Material). Further, the incubation of PTZ with the mitochondrial suspension did not result in GSH oxidation (Fig. 3B), suggesting that the reactivity of PTZ with protein thiol groups exhibits somehow a specificity probably due to the hydrophobicity of these drugs that become partitioned preferentially in membranes [57]. Data obtained from mass spectroscopy analysis showed that PTZ did not react spontaneously with GSH in the dark in a buffered aqueous medium (not shown).

To investigate the cause–consequence relationship between protein thiol oxidation and the opening of the PTP by PTZ, the effects of MPT modulators on the oxidation of mitochondrial protein thiol groups were evaluated. As shown in Fig. 3C, none of the modulators evaluated (DTT, GSH, NEM, CsA, Mg^{2+}, and EGTA) were able to inhibit the TR-induced thiol oxidation of mitochondrial proteins. Instead of protection, NEM amplified the TR-induced thiol oxidation of mitochondrial proteins probably by increasing the exposition of such groups of facilitating the access of TR to the target thiol. Such effect of NEM will be further investigated.

In this case, the lack of effect constituted an important finding: the absence of thiol oxidation inhibition by CsA, which partially and totally inhibited TR-induced mitochondrial swelling, suggested that thiol oxidation occurred before the opening of the PTP. This hypothesis was confirmed because EGTA failed to inhibit the oxidation of thiol groups but completely inhibited PTZ-induced MPT. This demonstrated that Ca^{2+} ions are not required for thiol oxidation by PTZ, but that they are indispensable to the opening of the permeability transition pore induced by these drugs.

3.4. Reactive oxygen species are not involved in PTZ-induced MPT

The direct oxidizing action of reactive oxygen species (ROS) on reduced mitochondrial membrane protein thiol groups resulting in thiol cross linkage and the opening of the PTP is well established [53,54]. In the particular case of PTZ-induced MPT, pre-incubating RLM with lipophilic α-tocopherol (vitamin E) and hydrophilic butylhydroxytoluene (BHT) antioxidants did not prevent swelling or the thiol oxidation elicited by PTZ (not shown). Also, as related previously, GSH was not consumed or oxidized during the incubation of RLM with PTZ. These findings suggest that PTZ-induced thiol oxidation related to the MPT trigger in the presence of Ca^{2+} was not directly promoted by ROS generated in the system. To confirm this hypothesis, electron paramagnetic resonance spectroscopy studies in isolated mitochondria using the spin trap DMPO (5,5-dimethyl-1-pyrroline-N-oxide) that reacts with superoxide anions or hydroxyl radicals resulting in DMPO adducts with distinguishable EPR signals was performed [58]. The incubation of TR with mitochondrial suspension under swelling assay conditions in the presence of DMPO resulted in no detectable EPR signal, similarly to the observed in the control, i.e., mitochondrial suspension in the absence of PTZ (not shown, Supplementary Material). Thus, the lack of swelling inhibition by antioxidants and the absence of a DMPO adduct EPR signal exclude the participation of oxygen-derived radicals in the oxidation of mitochondrial protein thiol groups and, consequently, MPT induced by PTZ.

In an attempt to investigate the oxidation of mitochondrial proteins, particularly the thiol groups, the spin trap DBNBS was used to detect possible formation and involvement of a protein-derived radical. Although some protein-derived radicals are sufficiently stable to be detected by direct EPR [59], no signal was directly detected from the incubation of RLM and TR or of only RLM in the absence of the spin trap (not shown). When the incubations were performed in the presence of the spin trap DBNBS that is capable of trapping water soluble carbon-centered radicals [60], DBNBS/$^{\bullet}$C-protein radical adducts were detected in basal conditions (only mitochondria) that were exacerbated significantly by TR (not shown). To test whether the effect was triggered by DBNBS and not by TR, the direct action of this spin trap on RLM was investigated. The addition of DBNBS to mitochondrial suspension promoted an extensive swelling *per se*, therefore preventing its utilization as a spin trap in EPR free radical investigations in isolated mitochondria. The specific effect of DBNBS on isolated mitochondria will be further investigated.

3.5. Investigation of the reactivity of TR with thiols in model systems

It has been previously proposed that PTZ-derived free radicals may be generated chemically by the action of peroxidases [61]. That cation radicals derived from PTZ can be photochemically generated in homogeneous and heterogeneous systems has also been shown [50]. Since mitochondria contain peroxidases and other proteins with peroxidase-like activity, it was hypothesized that the generation of the PTZ cation radical in isolated RLM could be responsible for the thiol oxidation observed and, consequently, MPT. To investigate this, Thimet oligopeptidase (TOP, MW 78 kDa) was selected as a model system due to its known number of –SH residues [62]. Also, the oxidation of these thiol groups results in dimer, trimer or even oligomer formation that is distinguishable in the SDS-PAGE by the increase of the intensity of bands above 156 kDa. Thus, to evaluate the reactivity of the TR ground state and the photochemically generated TR cation radical with the thiol groups of this model protein, TOP was partially reduced to give it free cysteine residues susceptible to possible PTZ attack. The TOP was then incubated in the dark and under irradiation in aqueous buffered medium with TR. After that, the reduced thiol groups were quantified using Ellman's reagent and an aliquot portion of protein was submitted to electrophoresis, as described.

As shown in Fig. 4A, –SH quantification revealed a significant decrease in the number of reduced TOP thiol groups incubated with TR under irradiation but not in the dark (i.e., the presence of TR and absence of irradiation). This suggests an oxidation of these groups by the PTZ-derived cation radical that was, in this case, photochemically generated. Also, electrophoresis of TOP samples revealed significant oligomerization when irradiated in the presence of TR, represented by an increase in the intensity of bands with high molecular weight (>156 kDa) (Fig. 4B, line d), which was not observed with TR in the dark (line c) or in the control (line b). In this experiment it was not possible to distinguish the formation of the TOP dimers or trimers specifically, but the generalized increase of the bands above 156 kDa referred to oligomerization by the action of PTZ cation radicals photochemically generated was clearly observed. Differences between in-the-dark and irradiated controls in the absence of TR were not observed.

To investigate the participation of a TR-derived cation radical possibly chemically generated by mitochondrial peroxidases, the peroxidase activities of respiratory complexes and other peroxidases were inhibited by associating cyanide with the blockage of electrons entering the respiratory chain. Such inhibitors were added to the mitochondrial suspension after the energization of mitochondria by succinate to allow calcium uptake. Afterward, mitochondrial swelling triggered by TR was estimated. Under this condition, TR failed to promote mitochondrial swelling (Fig. 4C) when compared to the control experiment in the absence of inhibitors suggesting the participation of chemically generated PTZ cation radicals in the oxidation of thiol groups and consequently MPT.

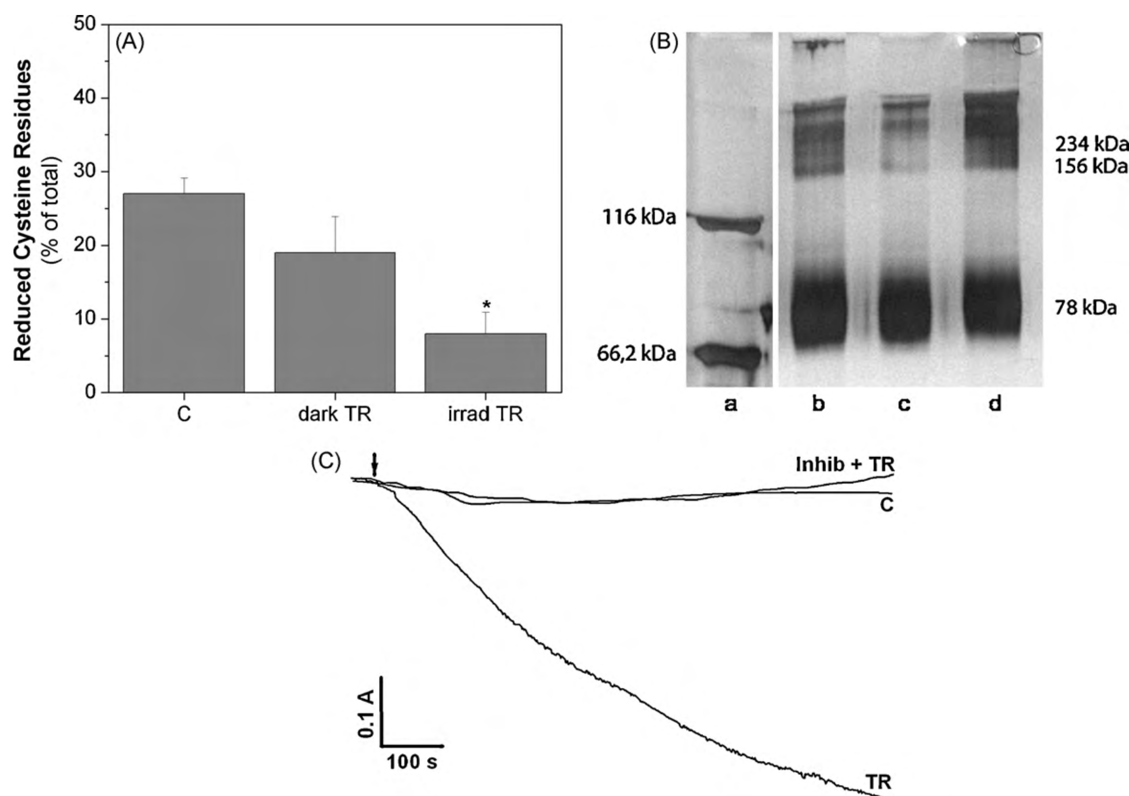


Fig. 4. Reactivity of thioridazine (TR) with Thimet oligopeptidase EC24.15 (TOP) and the effect of inhibition of mitochondrial peroxidases on TR-induced swelling. (A) After incubation of TOP with TR in the dark and under irradiation for 15 min, thiol groups were quantified using DTNB and the reduced cysteine residues were determined as described in Section 2. Bars: C, control, 40% reduced TOP in the absence of TR; dark TR, 1.5 μM TOP plus 4.5 μM TR kept in the dark; irrad TR, 1.5 μM TOP plus 4.5 μM TR under irradiation with a 4-W lamp (Model UVGL-25 Multiband UV) at 254 nm. (*) Statistically different from C ($p < 0.005$). (B) Samples obtained in (A) were submitted to a SDS-PAGE. Line a, standard molecular weight; line b, 40% reduced TOP in the absence of TR; line c, TOP plus TR in the dark; line d, TOP plus TR under a 15 min irradiation. (C): mitochondrial swelling measured in conditions described in Fig. 1. Lines: C, control in absence of TR; TR, 100 μM thioridazine; Inhib + TR, after calcium uptake mitochondria were inhibited by the addition of 1 mM KCN and 1 mM malonate followed by addition of TR (arrow) and the turbidity was measured.

3.6. *Cytochrome c release induced by thioridazine*

Currently, the involvement of mitochondria in the trigger and/or regulation of necrotic and apoptotic cell death is recognized [1,52]. Literature data show that mitochondria are able to release pro-apoptotic proteins to cytosol associated to MPT occurrence. One of these proteins is cytochrome *c*, which binds to Apaf-1, dATP and pro-caspase 9 to form an apoptosome with cleavage and activation of a caspase cascade in whole cells. However, it was also proposed that cyt *c* may be released independently of MPT occurrence [63]. To verify whether the PTZ-induced MPT in isolated rat liver mitochondria was associated to cyt *c* release, the supernatant cyt *c* amount was quantified using an enzyme-immune assay. Under the same experimental conditions as the swelling assays, TR induced the release of cyt *c* from mitochondrial suspensions to the supernatant, relative to the control experiment performed in the absence of the drug (Fig. 5). CsA also promoted partial inhibition of cyt *c* release, demonstrating that such release was due to MPT onset. Such MPT-associated-cyt *c* release in the presence of PTZ may be involved in the trigger of cell death induced by PTZ [32,33].

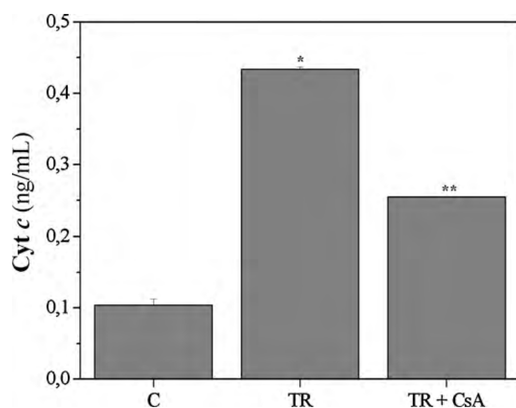


Fig. 5. Cytochrome c release induced by TR. After a 10 min of incubation under the same experimental conditions of the mitochondrial swelling assay described in Fig. 1, mitochondrial suspension was centrifuged at $16{,}000 \times g$ for 5 min. The amount of cyt *c* released was quantified in the supernatant by an enzyme-immune assay based method. Results were presented as mean ± s.d. of three experiments with different mitochondrial preparations. Bars: C, control in the absence of TR; TR, 100 μM thioridazine; TR + CsA, pre-incubation of mitochondria with 1.0 μM CsA and addition of 100 μM TR after energization. (*) Statistically different from C and (**) statistically different from TR ($p < 0.001$).

4. Discussion

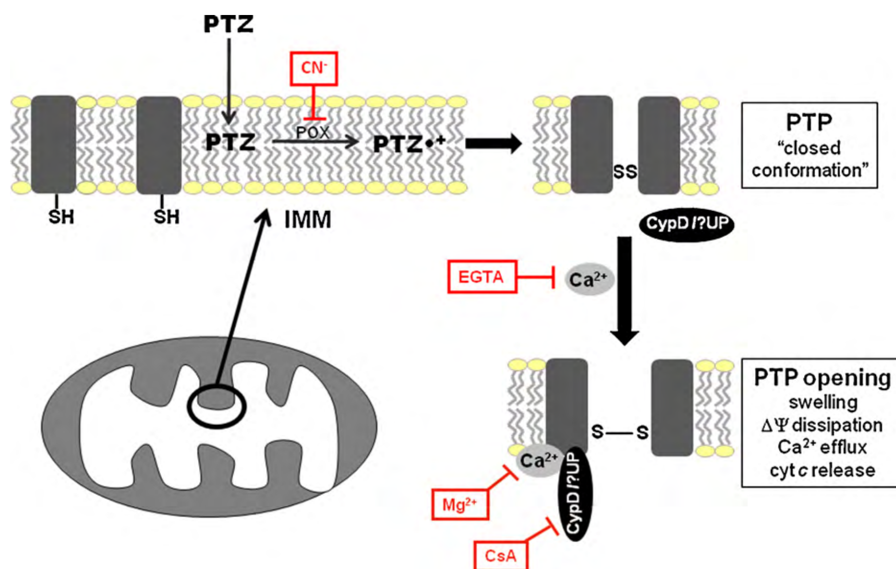
In 1979, the mitochondrial permeability transition phenomenon was described by Hunter and Haworth [3]. Since that time, MPT has been the focus of several studies due to its pivotal role in cell death and human diseases. Many advances have been made due to concentrated efforts by world-wide research groups who have tried to document the molecular composition and function of the PTP [64,65], although its exact molecular nature remains controversial. Studies regarding the underlying mechanisms of toxicity involving mitochondrial dysfunctions for some classes of drugs are relevant [66], but the use of drugs, independently of its therapeutic function, as a tool to investigate mechanistically the MPT is quite interesting.

Trifluoperazine, a piperazine phenothiazine derivative, has been described as a calmodulin antagonist [67,68] and MPT inhibitor [48,69,70]. In fact, all studies involving PTZ derivatives and mitochondrial function refer to their inhibitory effect on the PTP. However, the mechanism of MPT inhibition by TFP remains unclear. We previously contributed to this subject by showing that clinically prescribed phenothiazines (including TFP) at concentrations lower than 10 μM do not affect oxidative phosphorylation. In fact, in isolated rat liver mitochondria they display an important antioxidant activity against injury, induced by the prooxidants *tert*-butyl hydroperoxide and Fe^{2+}/citrate, that results in the inhibition of MPT and cyt *c* release [48]. These findings may contribute to explain the inhibition of apoptosis in CD4+ lymphocytes from HIV positive patients by PTZ [71].

However, several other studies showed that PTZ exhibit cytotoxic and antiproliferative activity in cultured cells [32,72]. Since mitochondrial permeability transition is strictly related to cell death, an investigation at the molecular level of the effects of PTZ on isolated liver mitochondria may be relevant to an understanding of mechanisms involved in drug-induced cytoxicity. Besides the use of drugs and chemicals that modulates MPT is an important tool to contribute for the understanding of the MPT process. Thus, the aim of this study was to elucidate the molecular mechanisms of MPT induction by phenothiazines in isolated rat liver mitochondria. It was shown that thioridazine, fluphenazine and trifluoperazine phenothiazine derivatives at higher concentrations were able to induce MPT in isolated mitochondria instead of inhibiting it. This result will likely change the approach to and use of these drugs as inhibitors of MPT in future studies, since they present a dual effect on MPT depending on their concentration. This not constitutes a contradictory effect due to the great variation at PTZ concentration (10-fold) between previous and the present study. Such effect inversion had already been described for other drugs [73,74].

The partial inhibition of MPT by CsA evidences the formation of a regulated PTP with the participation of CypD. It has been proposed that CyPD binds to the ANT and then, in the presence of Ca^{2+}, promotes a conformational change to induce the PTP formation [75]. According to Lemasters' group, when protein aggregates exceed the content of chaperones available to inhibit the pre-formed pores, PTP occurs in an unregulated way insensitive to CsA inhibition [76]. Thus, the stimulus promoted by PTZ to the opening of the mitochondrial transition pore has an unregulated component and the populations of protein complexes formed probably exhibit heterogeneity. The effect of CsA on related mitochondrial processes such as dissipation of the electrochemical gradient and Ca^{2+} efflux demonstrates that they occur as a consequence of the PTP opening. On the other hand, the lack of inhibition by CsA of the PTZ-promoted thiol modification demonstrates that such oxidation occurs before of the PTP opening. Additionally, the inhibition of the PTZ-induced mitochondrial swelling but not the thiol oxidation by EGTA demonstrates that the action of Ca^{2+} in the MPT process is downstream the thiol oxidation that is not sufficient *per se* to trigger MPT. A schematic representation is proposed (Scheme 1).

The current study shows that PTZ-induced MPT is dependent on the chemical modification of mitochondrial membrane protein thiol groups. The data support the hypothesis that such oxidation may be promoted by a chemically generated PTZ-derived cation radical. However, modifications were observed, specifically in thiol residues of the mitochondrial proteins, while GSH content was unaffected. Such findings show that PTZ somehow exhibit specificity for these protein thiols, indicating the necessity for a special microenvironment to allow the formation of PTZ cation radicals and their reaction with specific mitochondrial membrane protein thiols. In fact, the interaction of PTZ with biological membranes and lipid vesicle models is well known [31]. Further, these amphipathic molecules may become protonated and then positively charged at physiological pH, resulting in their accommodation near the hydrophobic/hydrophilic interface of micelles; also, such interaction is stronger with negative micelles [77]. Inner mitochondrial membranes possess a high content of the special negatively charged diphospholipid cardiolipin that enables them to accumulate lipophilic cations such as phosphonium derivatives and polyamines [78–80]. Such chemical characteristics of PTZ derivatives seem to contribute to the observed specificity in relation to the oxidation of thiol groups. In addition, the high efficiency of PTZ in promoting thiol oxidation and MPT without



Scheme 1. Proposed mechanism of induction of mitochondrial permeability transition by phenothiazines in isolated rat liver mitochondria. Phenothiazine (PTZ) interacts with inner mitochondrial membranes (IMM) being oxidized by mitochondrial peroxidases (POX) and generating the phenothiazine cation radicals ($PTZ^{\bullet+}$). These species modifies protein thiol groups of mitochondrial membranes resulting in a few protein aggregates associated to increased affinity of cyclophilin D and other unknown proteins (CypD/?UP) to the PTP complex that in the presence of Ca^{2+} results in PTP opening and associated processes. Also, it is represented the inhibition points of cyanide (CN^-), EGTA, Mg^{2+}, and cyclosporine A (CsA).

extensive protein aggregation suggests that PTZ promote the modification of specific thiol groups involved in the formation of PTP, contrary to the extensive high molecular weight aggregate formation observed in extensive stress oxidative conditions [56] that may involve other proteins than those thought to be involved in the PTP formation. Such specificity represents an important tool for future MPT studies.

Mitochondrial Ca^{2+} uptake is driven by the mitochondrial transmembrane potential and, in the matrix, is primarily important to the positive allosteric modulation of oxidative phosphorylation and other enzymes of oxidative metabolism [81–83]. However, at high Ca^{2+} concentrations or in the presence of prooxidants, metals, xenobiotics or drugs, mitochondria can undergo MPT, which is accompanied by extensive Ca^{2+} efflux. However, MPT can also occur in the absence of Ca^{2+} [84]. It was shown that palladacycles promote both thiol oxidation and MPT in isolated mitochondria independent of the presence of Ca^{2+} [55], but differently, the oxidation of mitochondrial thiol groups by PTZ is not sufficient to trigger the opening of the PTP in the absence of Ca^{2+}. It was proposed that chemicals able to promote the opening of the PTP may act only by decreasing the threshold for Ca^{2+}-induced MPT or act synergistically [52]. An important feature that deserves distinction is that Ca^{2+} ions inside the mitochondrial matrix are strictly necessary to link thiol oxidation promoted by PTZ to the MPT onset.

Ca^{2+} was thought to increase the binding of CypD to the matrix side of the protein complex resulting in PTP opening [85]. However, the partial inhibition by CsA suggests that other proteins insensitive to this inhibitor may be involved in the pore assembly. This will be further investigated.

Although the physiological relevance of MPT is unclear, related studies have had particular impact in recent years due to the capacity for MPT induction by a large number of drugs related to tissue toxicity. By taking place in whole cells, the induction of MPT by phenothiazines may impair ATP synthesis and promote the activation of cytosolic enzymes contributing to tissue injury and cell death. Corroborating the proposal that MPT induction may contribute to cyto- or tissue toxicity promoted by PTZ, mitochondrial permeabilization was accompanied by cyt *c* release. Although the role of cyt *c* and other mitochondrial pro-apoptic proteins is relatively well defined, the mechanisms for pro-apoptotic protein release and cell death regulation are still controversial [86].

Cardiolipin is present at higher concentrations in the inner mitochondrial membrane being the most likely candidate to interaction with cyt *c*. Since detachment of cyt *c* is involved with apoptosis trigger, the interaction between cardiolipin and cyt *c* has been extensively investigated [87]. It was proposed that a specific pore formed by Bax and other proteins allows the release of cyt *c* without mitochondrial swelling [63]. However, it was also shown that CsA is able to inhibit Bax-mediated apoptosis and cyt *c* release in cells, suggesting crosstalk between PTP and Bax-mediated pores [88]. It was recently proposed that Bax and MPT act cooperatively to cyt *c* release [89]. Since PTZ triggered MPT with cyt *c* release in isolated mitochondria, the participation of mitochondrial alterations in PTZ-induced cell death will be further investigated. Also, mitochondrial damage induced by PTZ may be implicated in cell death and tissue toxicity associated with the chronic intake of these drugs.

5. Conclusion

In summary, these results shows that clinically prescribed PTZ bury in the inner mitochondrial membranes and the chemically generated PTZ cation radicals modify specific thiol groups that in the presence of Ca^{2+} result in the trigger of the mitochondrial permeability transition associated to cytochrome *c* release. These findings contribute for: (i) the understanding of the molecular mechanisms involved in the MPT induction, (ii) the elucidation of the toxicity presented by these drugs, and (iii) may have implications for the cell death induced by PTZ.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.06.052.

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