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Photo-oxidative damage to isolated rat liver mitochondria induced by phenothiazines

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ABSTRACT

Photosensitization is a well-known side-effect of phenothiazines that could involve photochemically promoted oxidative damage to mitochondria, leading to the impairment of metabolic functions and apoptosis. In this work, for the first time, we investigated the effects of photoexcited thioridazine (TR), trifluoperazine (TFP) and fluphenazine (FP) on isolated rat liver mitochondria. Under UV irradiation, the presence of these phenothiazines led to a dose-dependent lack of the respiratory control ratio. These effects were not accompanied by significant swelling and oxidation of protein thiol groups but were accompanied by lipid peroxidation. Lycopene and sorbate, well-known quenchers of singlet oxygen and triplet species, respectively, were ineffective at protecting mitochondrial lipids against the damage promoted by the excited phenothiazines, suggesting that photochemically-produced cation radicals were the pro-oxidant species. Corroborating this proposal, butylated hydroxytoluene (BHT) completely inhibited the lipid peroxidation induced by UV irradiation in the presence of phenothiazines. These novel results make a significant contribution to the understanding of the photochemical properties of phenothiazines in biological systems.

Keywords: Trifluoperazine, thioridazine, fluphenazine, rat liver mitochondria, oxidative stress, photochemistry, photodamage, respiratory chain.

RESUMO

Dano foto-oxidativo em mitocôndrias de figado de rato induzido por fenotiazinas

A fotosensibilização é um efeito colateral bem conhecido das fenotiazinas e poderia envolver danos oxidativos em mitocôndrias promovidos fotoquimicamente levando à perda das funções metabólicas e apoptose. Neste trabalho, pela primeira vez, nós avaliamos os efeitos de tioridazina (TR), trifluoperazina (TFP) e flufenazina (FP) sobre mitocôndrias isoladas de fígado de rato. Sob irradiação, as fenotiazinas investigadas levaram à perda da razão do controle respiratório de uma forma dose-dependente. Estes efeitos não foram acompanhados por significativo inchamento mitocondrial e oxidação de grupos tiol de proteínas mas foram acompanhados por peroxidação lipídica. Licopeno e sorbato, supressores bem conhecidos de oxigênio singlete e espécies tripletes, respectivamente, foram ineficientes para proteger os lipídios mitocondriais contra o dano promovido pelas fenotiazinas excitadas, sugerindo que os cátions radicais produzidos fotoquimicamente foram as espécies oxidantes. De acordo com esta proposta, BHT inibiu completamente a peroxidação lipídica induzida pela irradiação UV na presença de fenotiazinas. Estes novos resultados constituem importante contribuição para a compreensão das propriedades fotoquímicas das fenotiazinas em sistemas biológicos.

Palavras-chave: Trifluoperazina, tioridazina, flufenazina, mitocôndrias de fígado de rato, estresse oxidativo, fotoquímica, fotodano, cadeia respiratória.

INTRODUCTION

Phenothiazines are heterocyclic compounds formed out of two benzene rings linked by one sulfur and one nitrogen atom, belonging to the class of thiazine dyes, such as methylene blue, whose photochemical properties have already been described. The photochemical behavior of phenothiazine derivatives has gained interest because compounds containing a phenothiazine moiety may promote photosensitizing effects in patients treated with these drugs, which are widely used for schizophrenia treatment (Elisei et al., 2002; Viola et al., 2003).

Mitochondria are organelles within are located the metabolic pathways and respiratory chain responsible for the synthesis of ATP coupled to the oxidation of energy-rich substrates by molecular oxygen (Boyer et al., 1977; Mitchell, 1979; Brown, 1992). The use of molecular oxygen by mitochondria of eukaryotic cells was an important evolutive acquisition since allowed a higher energetic yield from the metabolic fuels. However, a fraction of the molecular oxygen available to the respiratory chain undergoes incomplete reduction, generating the superoxide radical anion, which is promptly dismutated to hydrogen peroxide (Boveris & Chance, 1973; Gonzales-Flecha & Boveris, 1995). Hydrogen peroxide reacts with Fe(II) by a mechanism known as the Fenton reaction, generating the hydroxyl radical, the most reactive species generated in living cells (Halliwell & Gutteridge, 1999). The superoxide radical anion can also react with nitric oxide, generating peroxynitrite, a powerful pro-oxidant species. These reactive oxygen and nitrogen species are prone to attack DNA, phospholipids and proteins. (Blei, 1977; Piette et al., 1986). Therefore, mitochondria are concomitantly the source and target of these reactive species in processes related to aging, mutagenesis and cancer (Singh, 2004).

In parallel with the oxidative stress produced by the incomplete reduction of molecular oxygen, the reactive species generated by photochemical processes can also cause oxidative stress in mitochondria. This photo-oxidative stress is particularly significant during photodynamic therapy (PDT). (Henderson & Dougherty, 1992). In this regard, mitochondria have the ability to concentrate cationic sensitizers such as porphyrins and thiazine compounds (Bhowmik et al., 2001; Severino et al., 2003). The triplet excited states of these sensitizers lead to cell death due to oxidative stress produced by singlet oxygen (Type II mechanism) and free radicals (Type I mechanism) (Moore, 1977). The cell death promoted by

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PDT occurs via apoptosis and necrosis, processes in which mitochondria may take part either upstream or downstream (Martinou et al., 1999; Hengartner, 2000). This participation of mitochondria in apoptosis and necrosis involves impairment of the organelle functions, characterized by well-known processes such as uncoupling and respiratory chain inhibition. Both uncoupling and respiratory inhibition are related to the opening of a permeability transition pore (PTP) (Zoratti & Szabò, 1995), an event implicated in apoptosis triggered by cytochrome *c* release (Toescu, 1998; Susin et al., 1998). In this regard, cytochrome *c* can be converted to a high spin inactive form after irradiation in the presence of methylene blue (Estevam et al., 2004).

Although the photochemical behavior of phenothiazines has been extensively studied, literature lacks studies about the sensitizing effects of phenothiazines on biological systems, especially mitochondria (Eberlein-Konig et al., 1997; Elisei et al., 2002; Viola et al., 2003). In this work, for the first time, we evaluate the effects of photoexcited thioridazine (TR), trifluoperazine (TFP) and fluphenazine (FP) on isolated rat liver mitochondria to contribute for the comprehension of the photo-triggered side effects of phenothiazine therapy.

MATERIALS AND METHODS

Thioridazine, trifluoperazine and fluphenazine were purchased from Sigma Chemical Co. (St Louis, MO, USA). All aqueous solutions were prepared with deionized water (mixed-bed ion exchanger, Millipore) and the pH was measured with a combined glass electrode (Orion Glass pH SURE-FLOW™). The reference electrode (ROSS™, model 8102) was filled with Orion Filling Solution (ROSS™). The pH meter was calibrated using METREPAK pHydrion standard buffer solutions (Brooklyn, NY). Phenothiazine-loaded systems were irradiated with a 4-Watt lamp, Model UVGL-25 Multiband UV 254/365 nm.

Isolation of rat liver mitochondria. Mitochondria were isolated by standard differential centrifugation (Cain & Skilleter, 1987). Male Wistar rats weighing approximately 200 g were sacrificed by cervical dislocation. The liver was immediately removed, sliced in approximately 35 mL of medium containing 250 mM sucrose, 1 mM EGTA and 10 mM HEPES-KOH, pH 7.2, and homogenized three times for 15 sec at 1 min intervals with a Potter-Elvehjem homogenizer. This homogenate was centrifuged at 580×*g* for 5 min and the resulting supernatant was further centrifuged at 10,300×*g* for 10 min. Pellets were suspended in 10 mL of medium containing 250 mM sucrose, 0.3 mM EGTA and 10 mM HEPES-KOH, pH 7.2, and centrifuged at 3400×*g* for 15 min. The final mitochondrial pellet was suspended in 1 mL of medium containing 250 mM sucrose and 10 mM HEPES-KOH, pH 7.2. For all assays, the mitochondrial preparation was used within 3 h and mitochondrial protein content was determined by the Biuret reaction.

Mitochondrial respiration. Mitochondria (1 mg/mL) were incubated in 2 mL of medium containing 125 mM sucrose, 65 mM KCl, 0.5 mM EGTA, 10 mM K_2HPO_4 and 10 mM HEPES-KOH, pH 7.2, at 30 °C. Mitochondrial respiration was monitored polarographically by an oxygraph equipped with a Clark-type oxygen electrode (Gilson Medical Electronics, Middleton, WI, USA). State 4 respiration was initiated by adding potassium succinate to a final concentration of 5mM (+ 2.5 μM rotenone) after which 400 nmol of ADP were added to initiate state 3 respiration. The respiratory control ratio (RCR) was calculated as the ratio of state 3 and state to 4 respiration rates (Chance & Willians, 1956; Mingatto et al., 1996).

Mitochondrial swelling. Mitochondria (0.4 mg protein) were incubated in 1.5 mL of a medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.2, 5 mM potassium succinate (+ 2.5 μM rotenone) and 10 μM $CaCl_2$ at 30 °C, in the presence and absence of phenothiazines. The extent of mitochondrial swelling was determined from the difference between initial (zero time) and final (after 15 min) absorbance at 540 nm in a Hitachi U-2000 Spectrophotometer (Tokyo, Japan).

Determination of protein-thiol content. After 15 min incubation with phenothiazines under swelling conditions, mitochondria were treated with trichloroacetic acid (5 % final concentration) and centrifuged at 4500×*g* for 10 min. The pellet was suspended with 1 mL of 0.5 M potassium phosphate buffer, pH 7.6, and, after addition of 0.1 mM DTNB, absorbance was determined at 412 nm. The amount of thiol groups was calculated from ε = 13, 600 M^{-1} (Jocelyn, 1987).

Lipid peroxidation assay. Lipid peroxidation (LPO) of mitochondrial membrane was measured as MDA generation. Mitochondria (1 mg/mL) were incubated in the presence or absence of phenothiazines in a medium containing 130 mM KCl, 10 mM HEPES-KOH, pH 7.4, and 5 mM potassium succinate (+ 2.5 μM rotenone) for 30 min at 37 °C. As positive control, it was used 50 μM $(NH_4)_2Fe(SO)_4$ and 2 mM sodium citrate. To determine MDA, 1 mL of 1 % TBA (prepared in 50 mM NaOH), 0.1 ml of 10 M NaOH and 0.5 mL of 20 % H_3PO_4 were added, followed by further incubation for 20 min at 85°C. The MDA-TBA complex was extracted with 2 mL of *n*-butanol and the absorbance measured at 532 nm. MDA concentration was calculated from ε = $1.56x10^5$ M^{-1} (Buege & Aust, 1978).

RESULTS

Effects of irradiated phenothiazines on the respiratory control ratio of rat liver mitochondria

The effects of TR, TFP and FP on the respiratory control ratio (RCR) of rat liver mitochondria were determined over a concentration range of 1-25 μM, under UV irradiation and in the dark. In the above conditions, the samples were incubated during 10 min at 25°C prior the addition of succinate, in the presence of rotenone. The

effects of TR, TFP and FP on rat liver mitochondria, in the dark and over the same concentration range have previously been published (Pereira et al., 1992; Rodrigues et al., 2002). In these works it was shown that TR, TFP and FP, added to respiring rat liver mitochondria, did not affect the RCR significantly and were able to inhibit the opening of PTP induced by *t*-BuOOH or Fe^{2+}/citrate. In the present experimental conditions, a similar effect on rat liver mitochondrial RCR was observed when these organelles were incubated with phenothiazines in the dark. However, when the mitochondria were incubated with phenothiazines for 10 min at 25°C, under irradiation at 365 nm with a 4-Watt UV lamp, it was detected increase of state 4 respiration rate (uncoupling) in a phenothiazine dose-dependent manner (Figure 1, left panel). A significant decrease in the state 3 respiration rate under irradiation, was observed only in the presence of 25 μM TFP and FP (Figure 1, right panel). The inset in Figure 1 shows a representative kinetic profile of oxygen consumption by rat liver mitochondria after 10 min of incubation under UV-irradiation in the absence of phenothiazines (line A), in the presence of 5 μM TR (line B) and in the presence of 25 μM TR (line C).

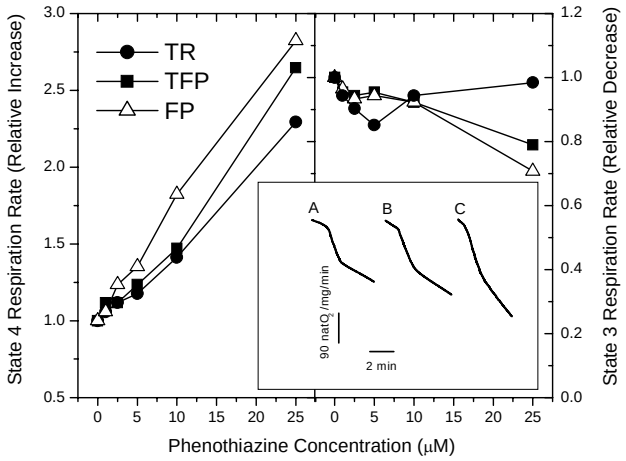


FIGURE 1 - Concentration-response curves for the effects of irradiated thioridazine (TR) (filled circle), fluphenazine (FP) (open triangle) and trifluoperazine (TFP) (filled square) on the state 4 and state 3 respiratory rates of isolated rat liver mitochondria. Mitochondria (1.0 mg protein) were incubated at 30°C with 5 mM succinate and 2.5 μM rotenone in a standard respiration medium containing 125 mM sucrose, 65 mM KCl and 10 mM HEPES-KOH, pH 7.2, in the presence of 0.5 mM EGTA and 10 mM K_2HPO_4 in a final volume of 1.8 mL. State 3 respiration was initiated with 400 nmol ADP. For the irradiated control state 4 respiration rate was 21.20 ± 0.21 natO/min/mg protein and state 3 respiration rate was 121.60 ± 2.40 natO/min/mg protein. In the dark, the control state 4 respiration rate was 19.88 ± 1.25 natO/min/mg protein and the state 3 respiration rate was 130.88 ± 3.08 natO/min/mg protein. Data are presented as the means +/- s.e.m. of three experiments with different mitochondrial preparations. The inset shows the kinetic profile of oxygen consumption by rat liver mitochondria after 10 min of incubation under UVirradiation in the following conditions: A) no phenothiazine, B) presence of 5 μM TR and C- presence of 25 μM TR.

The increase in state 4 respiration rate (uncoupling) led to a decrease in RCR, consequently also in a dose-dependent manner (Figure 2). In the experimental conditions used here, significantly RCR alteration (> 10%) in the dark was detected only in the presence of phenothiazine concentrations ≥ 10 μM (Figure 2 open circle). In the absence of phenothiazines, the UV irradiation produced a slight decrease in RCR (up to 10%, not shown). As expected, under these conditions, in which EGTA was present, rat liver mitochondria did not exhibit swelling (data not shown). In the presence of 20 μM Ca^{2+}, a slight turbidity decrease (at most 10%) was detected in both the presence and absence of phenothiazines (data not shown).

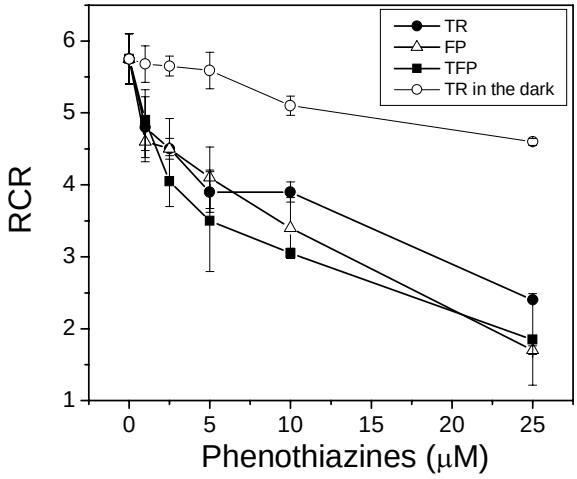


FIGURE 2 - Concentration-response curves for the effects of irradiated thioridazine (TR) (filled circle), fluphenazine (FP) (open triangle), trifluoperazine (TFP) (filled square) and thioridazine (TR) in the dark (open circle) on the RCR of isolated rat liver mitochondria. Experimental conditions are as for Figure 1.

Effects of irradiated phenothiazines on the mitochondrial proteins and lipids

Considering that the uncoupling and the decrease in the mitochondrial RCR (Figures 1 and 2) induced by irradiated phenothiazines were not accompanied by mitochondrial swelling (data not shown), the lipid fraction of the organelle seems to be the most probable target for the observed oxidative damage. In this regard, the mitochondrial damage was not accompanied by a significant (Castilho et al., 1996) decrease in the proportion of reduced protein thiol groups (Figure 3), but was accompanied by a significant increase in the malondialdehyde content (MDA), which indicates oxidation of the mitochondrial membrane lipid acyl chains (Figure 4). In order to identify the main pro-oxidant species involved in the photodamage promoted by irradiated phenothiazines, sorbate, a quencher of triplet excited states, lycopene, a quencher of singlet oxygen, and BHT, which traps free radicals, were tested for ability to prevent photodamage. Only BHT was able to prevent the LPO induced by irradiated phenothiazines (Figure 4).

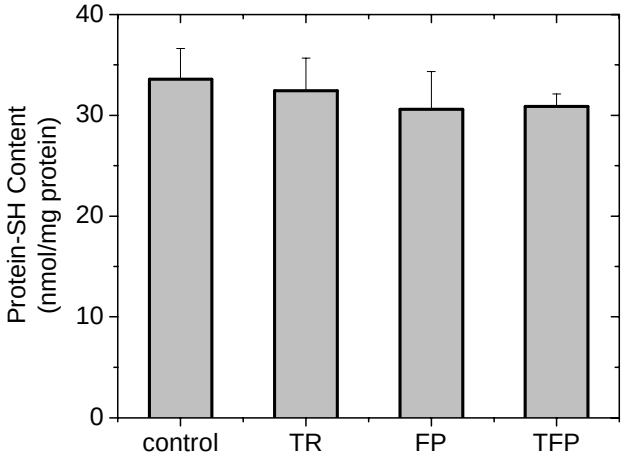


FIGURE 3 - Effects of 25 μM thioridazine (TR), fluphenazine (FP) and trifluoperazine (TFP) on the oxidation of mitochondrial protein-SH. Mitochondria (0.4 mg protein) were incubated in the standard medium with 5 mM succinate and 2.5 μM rotenone, at 30°C (1.5 mL final volume). See Methods for other details. Data are presented as the mean +/- s.e.m. of three experiments with different mitochondrial preparations. Statistical analysis by impaired t-test was performed with GraphPad Prism 3.0 (GraphPad Software Inc, San Diego, CA). No statistical difference among samples was observed (P<0.05).

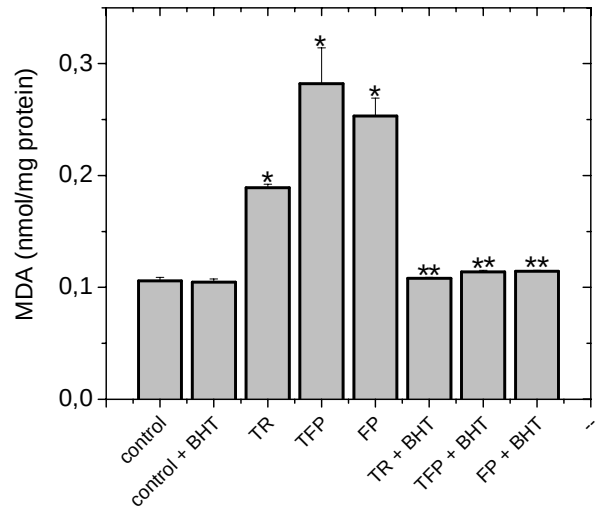


FIGURE 4 - Effects of 25 μM thioridazine (TR), fluphenazine (FP) and trifluoperazine (TFP) on lipid peroxidation assayed as MDA generation. Mitochondria (1 mg protein) were incubated in the standard medium with 5 mM succinate, 2.5 μM rotenone, 50 μM $(NH_4)_2Fe(SO_4)_2$ and 2 mM sodium citrate for 30 min at 37°C (1 mL final volume). See Methods for MDA determination. Data are presented as the mean +/- s.e.m. of three experiments with different mitochondrial preparations. Statistical analysis was performed by unpaired t-test, using GraphPad Prism 3.0 from GraphPad Software Inc. (San Diego, CA). * Significantly different from control (P<0.05); ** significantly different from * (P<0.05).

DISCUSSION

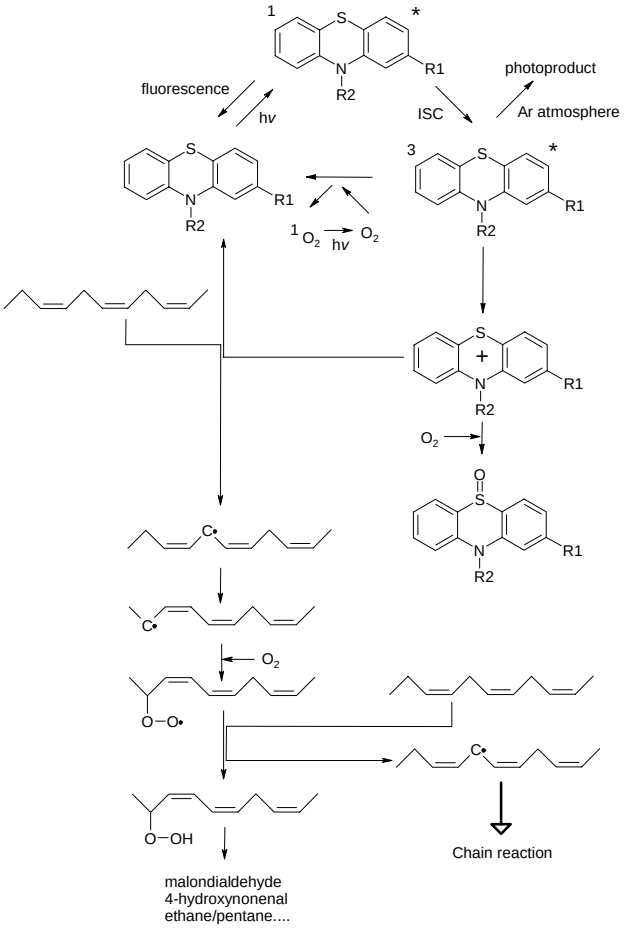
The photodamage promoted by irradiated phenothiazines led to a loss of mitochondrial transmembrane potential

The RCR shown in Figure 1 is the ratio of state 3 (energized) to state 4 (resting) respiration rates and it reflects the efficiency of the respiratory chain. Thus, the observed decrease in rat liver mitochondrial RCR could have been produced by stimulation of state 4 (uncoupling), inhibition of state 3, or both simultaneously. In the experimental conditions described here, the decrease in rat liver mitochondrial RCR was produced significantly by the increase of state 4 respiration rate (uncoupling) as significant decrease in the state 3 respiration rate was observed, under irradiation, only in the presence of 25 μM TFP and FP (Figure 2). The partial inhibition of the respiratory chain observed in the presence of higher concentrations of irradiated phenothiazines could be a consequence of damage to the respiratory chain components. In this regard, we have observed oxidative damage to cytochrome *c* promoted by excited states of 25 μM phenothiazines in both homogeneous and heterogeneous media (unpublished results). The increase in the state 4 respiration rate that was dependent of the phenothiazine concentration suggests damage on mitochondria able to dissipate the transmembrane potential ($\Delta\Psi$) without opening of the permeability transition pore (PTP), since the respiration rate was determined in the presence of 0.5 mM EGTA (Kowaltowski et al., 1996). The occurrence of mitochondrial permeability transition (MPT) due to the opening of the PTP was definitively discarded by the inability of irradiated phenothiazines to induce significant mitochondrial swelling in the presence of 20 μM Ca^{2+}. Thus, it was expected that the loss of the mitochondrial transmembrane potential could be caused mainly by oxidative damage to the mitochondrial lipid fraction, and this was corroborated by the significant increase in the MDA content observed after irradiation of rat liver mitochondria in the presence of phenothiazines (Figure 4). The efficiency with which irradiated phenothiazines induce damage to mitochondrial membranes is coherent with their high affinity for the inner mitochondrial membrane (unpublished results). In this case, the slight oxidative damage (Figure 3) observed for the mitochondrial protein fraction should not be enough to induce MPT.

The cause of the mitochondrial damage induced by irradiated phenothiazines

At this point it was important to determine the cause of the mitochondrial loss of function observed after irradiation in the presence of TR, TFP and FP. Under irradiation at 365 nm, phenothiazines are excited to the first singlet state ($S_0 \rightarrow S_1$ transition). This excited state suffers intersystem crossing to the first triplet state ($S_1 \rightarrow T_1$ crossing). The phenothiazine triplet states can transfer energy to molecular oxygen and generate singlet oxygen or react with another phenothiazine molecule and generates its cation radical (Martinez et al., 2004). Thus, there are three possible long-lived pro-oxidant species generated during the irradiation of TR, TFP and FP that could cause

mitochondrial damage: the triplet state, singlet oxygen and the cation radical. The inability of sorbate and lycopene to prevent mitochondrial damage induced by irradiated phenothiazines suggests that the cation radicals were the pro-oxidant species involved in the attack on the mitochondrial lipid fraction, leading to the decrease in RCR. Scheme 1 illustrates the proposed mechanism of lipid damage induced by excited phenothiazines. Accordingly, only BHT, a well known antioxidant for lipids, was able to prevent the LPO induced by irradiated phenothiazines (Figure 4).



SCHEME 1 - Proposed mechanism for the mitochondrial lipid damage induced by photoexcited phenothiazines.

Implications of this study in the pharmacological side effects of phenothiazines

It is well known that patients treated with phenothiazine drugs exhibit enhanced photosensitivity in skin exposed to sunlight. The data on rat liver mitochondria that show the ability of the pro-oxidant species generated during irradiation of phenothiazines to impair mitochondrial functions, especially those related to bioenergetics, suggest that such oxidative damage could be involved in the side-effects of this class of drugs.

In conclusion, the irradiation of phenothiazines, in a medium containing rat liver mitochondria, led to the formation of pro-oxidant species, mainly cation radicals that were able to promote mitochondrial injury, damaging mitochondrial lipids and promoting respiratory chain uncoupling.

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