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Hydroxytamoxifen protects against oxidative stress in brain mitochondria

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Abstract

This study evaluated the effect of hydroxytamoxifen, the major active metabolite of tamoxifen (synthetic, nonsteroidal antiestrogen drug), on the function of brain mitochondria. We observed that only high concentrations of hydroxytamoxifen (60 nmol/mg protein) induced a significant decrease in RCR, while ADP/O ratio remained statistically unchanged. Similarly, only the highest concentration of hydroxytamoxifen (60 nmol/mg protein) affected the phosphorylative capacity of brain mitochondria, characterized by a decrease in the repolarization level and an increase in the repolarization lag phase. We observed that all the concentrations of hydroxytamoxifen tested (7.5, 15 and 30 nmol/mg protein) prevented lipid peroxidation induced by the oxidant pair ADP/Fe^{2+}. Furthermore, through the analyses of calcium fluxes and mitochondrial transmembrane potential parameters, we observed that hydroxytamoxifen (30 nmol/mg protein) exerted some protection against pore opening, although in a less extension than that promoted by cyclosporin A, the specific inhibitor of the mitochondrial permeability transition pore. However, in the presence of hydroxytamoxifen plus cyclosporin A, the protection observed was significantly higher when compared with that induced by both agents alone.

These results support the idea that hydroxytamoxifen protects lipid peroxidation and inhibits the mitochondrial permeability transition pore in brain. Since numerous neurodegenerative diseases are intimately related with mitochondrial dysfunction resulting from lipid peroxidation and induction of mitochondrial permeability transition, among other factors, future therapeutical strategies could be designed taking in account this neuroprotective role of hydroxytamoxifen, which is pharmacologically much more potent and less toxic than its promoter tamoxifen.

Keywords: Brain; Calcium; Hydroxytamoxifen; Lipid peroxidation; Mitochondrial permeability transition; Neuroprotection

1. Introduction

Tamoxifen (TAM) is a non-steroidal estrogenic compound used for the treatment of breast cancer. It is a first-generation selective estrogen receptor modulator (SERM) and acts as an estrogen antagonist in mammary tissue but mimics the effects of estrogen in other tissues [1]. TAM undergoes extensive hepatic metabolism being hydroxytamoxifen (OHTAM) its major active metabolite and responsible for its antiestrogenic activity [2]. Besides their chemopreventing function, TAM and OHTAM, which are extremelly lipophilic [3,4], induce multiple cellular effects, including antioxidant actions since they are strong intramembranous scavengers of peroxyl radicals [5].

TAM and OHTAM can penetrate the blood brain barrier and interact with several neuronal estrogen receptor-positive cell populations [6,7]. Previous studies in vitro [8] showed that OHTAM has a higher affinity for the estrogen receptor than TAM. Furthermore, OHTAM is 100- to 200-times more potent [9,10] and it is pharmacologically well tolerated [11] and less toxic to erythrocytes [12] and to sarcoplasmic reticulum calcium pump [13]. Dietze and collaborators [14] also reported that OHTAM, in contrast to TAM, do not induce apoptosis in p53(−) human mammary epithelial cells. Although most studies of TAM and its metabolites have been focused on its ability to compete

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with estrogens for binding to its receptors, several recent studies have described additional estrogen receptor-independent activities of TAM [15,16].

Data from the literature point for a neuroprotective role associated to TAM and OHTAM being the study of TAM effects more explored. Previous studies have shown that TAM is effective in protecting brain tissue from rat models of reversible and permanent focal ischemia [17,18] and suppress the $HO^{\bullet}$ generation via dopamine efflux induced by MPP^{+} [19]. O'Neill and Brinton [20] showed that both TAM and OHTAM attenuate the excitotoxic glutamate-induced intracellular Ca^{2+} rise.

It has become clear that mitochondria have a significant role to play not only in energy production, but also in cell death [21]. A potentially central factor in cell death in neurodegeneration is the mitochondrial permeability transition pore (MPTP) [22,23]. The MPTP is a phenomenon that is characterized by the opening of pores in the inner mitochondrial membrane and by its sensitivity to a very low concentration of cyclosporin A (CsA). Ca^{2+} and oxidative stress have long been known to favor the permeability transition [24].

Previous studies indicate that TAM [25] and OHTAM [26] are potent inhibitors of the MPTP in liver mitochondria. Furthermore, Hoyt et al. [27] reported that TAM could be an inhibitor of the MPTP in intact neurons. Our first goal was the evaluation of the impact of increasing concentrations of OHTAM (15, 30, 60 nmol/mg protein) in the respiratory chain and oxidative phosphorylation system. For that purpose we analysed the respiratory indexes (RCR and ADP/O), mitochondrial transmembrane potential ($\Delta\Psi_m$), repolarization level, repolarization lag phase and ATP levels. The second part of this study was aimed to evaluate the protective effect of OHTAM in the lipid peroxidation induced by the pair ADP/Fe^{2+} and in the induction of MPTP by Ca^{2+}. Lipid peroxidation was evaluated by oxygen consumption and TBARS formation. MPTP induction was characterized by analyzing the mitochondrial $\Delta\Psi_m$, calcium fluxes and glutathione content.

2. Materials and methods

2.1. Materials

Hydroxytamoxifen and Protease type VIII (Subtilisin Carlsberg) were obtained from Sigma (St. Louis, MO). Digitonin was obtained from Calbiochem (Merck Biosciences Ltd., Nottingham). All the other chemicals were of the highest grade of purity commercially available.

2.2. Animals

Male Wistar rats (3 months old) were housed in our animal colony (Laboratory Research Center, University Hospital, Coimbra, Portugal). They were maintained under controlled light (12 h day/night cycle) and humidity with free access to water (except in the fasting period) and powdered rodent chow (URF1-Charles River, France). Adhering to procedures approved by the Institutional Animal Care and Use Committee, the animals were sacrificed by cervical displacement and decapitation.

2.3. Isolation of brain mitochondria

Brain mitochondria were isolated from male Wistar by the method of Rosenthal et al. [28], with slight modifications, using 0.02% digitonin to free mitochondria from the synaptosomal fraction. In brief, one rat is decapitated, and the whole brain minus the cerebellum was rapidly removed, washed, minced, and homogenised at 4 °C in 10 ml of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mM EGTA, 1 mg/ml bovine serum albumin, pH 7.4) containing 5 mg of the bacterial protease. Single brain homogenates were brought to 30 ml and then centrifuged at 2500 rpm (Sorvall RC-5B Refrigerated Superspeed Centrifuge) for 5 min. The pellet, including the fluffy synaptosomal layer, was resuspended in 10 ml of the isolation medium containing 0.02% digitonin and centrifuged at 10,000 rpm for 8 min. The brown mitochondrial pellet without the synaptosomal layer was then resuspended again in 10 ml of medium and recentrifuged at 10,000 rpm for 10 min. The mitochondrial pellet was resuspended in 300 μl of resuspension medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, pH 7.4). Mitochondrial protein was determined by the biuret method calibrated with bovine serum albumin [29].

2.4. Mitochondrial respiration measurements

Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode (YSI Model 5331, Yellow Springs Inst.) [30] connected to a suitable recorder in a 1 ml thermostated water-jacketed closed chamber with magnetic stirring. The reactions were carried out at 30 °C in 1 ml of the reaction medium (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 5 mM Hepes, 10 μM EGTA, pH 7.4) with 0.6 mg protein. The isolates were incubated with OHTAM for 2 min before succinate addition. ADP (125 nmol/mg protein) was added after energization of mitochondria.

2.5. Membrane potential ($\Delta\Psi_m$) measurements

The mitochondrial transmembrane potential was monitored by evaluating the transmembrane distribution of tetraphenylphosphonium (TPP^{+}) with a TPP^{+}-selective electrode prepared according to Kamo et al. [31] using an $Ag/AgCl_2$ electrode as reference.

Reactions were carried out in a chamber with magnetic stirring in 1 ml of reaction medium supplemented with 3 μM TPP^{+}. The experiments were started by adding 5 mM

succinate to mitochondria in suspension at 0.6 mg protein/ml. Membrane potential was estimated from the decrease on TPP^+ concentration in the reaction medium as described elsewhere [32]. The isolates were incubated with OHTAM for 2 min before succinate addition. In the MPTP experiments, after a steady-state distribution of TPP^+ had been reached (≈2 min of recording), Ca^{2+} was added and $\Delta\Psi_m$ recorded.

2.6. Analysis of ATP content

At the end of the $\Delta\Psi_m$ experiments, 250 μl of each mitochondrial suspension were rapidly centrifuged at 14,000 rpm (Eppendorf Centrifuge 5415C) for 4 min with 0.3 M perchloric acid. The supernatants were neutralised with 10 M KOH in 5 M Tris and centrifuged at 14,000 rpm (Eppendorf Centrifuge 5415C) for 4 min. The resulting supernatants were assayed for ATP by separation in a reverse-phase high performance liquid chromatography. The chromatography apparatus was a Beckman-System Gold, consisting of a 126 Binary Pump Model and 166 Variable UV detector controlled by a computer. The detection wavelength was 254 nm, and the column was a Lichrospher 100 RP-18 (5 μm) from Merck. An isocratic elution with 100 mM phosphate buffer (KH_2PO_4) pH 6.5 and 1.0% methanol was performed with a flow rate of 1 ml/min. The required time for each analysis was 6 min.

2.7. Lipid peroxidation

The extent of lipid peroxidation was evaluated by oxygen consumption using a Clark-type electrode (YSI Model 5331, Yellow Springs Inst.) in a closed glass chamber equipped with magnetic stirring, thermostated at 30 °C, as described by Dinis et al. [33]. Reactions were started by the addition of ADP/Fe^{2+}. The changes in O_2 tension were recorded in a potentiometric chart recorder and the oxygen consumption calculated assuming an oxygen concentration of 236 nmol O_2/ml. OHTAM was introduced in the incubation medium, containing mitochondria, 2 min before the addition of ADP/Fe^{2+}.

Lipid peroxidation was also determined by measuring thiobarbituric acid reactive substances (TBARS), using the thiobarbituric acid assay (TBA), according to a modified procedure described by Ernster and Nordenbrand [34]. The amount of TBARS formed was calculated using a molar coefficient of $1.56 \times 10^5\ mol^{-1}\ cm^{-1}$ and expressed as nmol TBARS/mg protein.

2.8. Calcium fluxes

Mitochondrial calcium fluxes were measured by monitoring the changes in Ca^{2+} concentration in the reaction medium using a calcium-selective electrode according to previously described procedures [32]. The reactions were conducted in an open vessel with magnetic stirring in 1 ml of the reaction medium containing 100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 10 μM EGTA, 5 mM Hepes (pH 7.4), with 2 μM rotenone. Mitochondria (0.8 mg/ml) were energized with 5 mM succinate after 1 min of Ca^{2+} addition. 30 nmol OHTAM/mg protein, 0.85 μM CsA and 2 μg/ml oligomycin plus 1 mM ADP were added to the reaction medium 2 min prior Ca^{2+} addition. In CsA plus OHTAM condition, CsA was added after mitochondria energization.

2.9. Glutathione content

Reduced (GSH) and oxidized (GSSG) glutathione were determined with fluorescence detection after reaction of the supernatant of the H_3PO_4/EDTA-NaH_2PO_4 or H_3PO_4/NaOH deproteinized mitochondria solution, respectively, with *o*-phthalaldehyde (OPT), pH 8.0, according to Hissin and Hilf [35]. In brief, at the end of the $\Delta\Psi_m$ experiments, 500 μl of each mitochondrial suspension were rapidly centrifuged at 50,000 rpm (Beckman, TL-100 Ultracentrifuge) for 30 min with 1.5 ml phosphate buffer (100 mM NaH_2PO_4, 5 mM EDTA, pH 8.0) and 500 μl H_3PO_4 2.5%. For GSH determination 100 μl of the supernatant were added to 1.8 ml phosphate buffer and 100 μl OPT. After mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette and the fluorescence was measured at 420 nm. For GSSG determination 250 μl of the supernatant were added to 100 μl of *N*-ethylmaleymide and incubated at room temperature for 30 min. After the incubation 140 μl of the mixture were added to 1.76 ml NaOH (100 mM) buffer and 100 μl OPT. After mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette and the fluorescence was measured at 420 and 350 nm emission and excitation wavelength, respectively (slits 5, 5). The GSH and GSSG contents were determined from comparisons with a linear reduced or oxidized glutathione standard curve, respectively.

2.10. Statistical analysis

Results are presented as mean ± S.E.M. of the indicated number of experiments. Statistical significance was determined by using the one-way ANOVA test for multiple comparisons, followed by the post hoc Tukey–Kramer test or by the unpaired two-tailed Student's *t*-test.

3. Results

3.1. Effect of OHTAM in brain mitochondrial respiration

Respiratory control ratio (RCR) is the ratio between mitochondrial respiration states 3 (consumption of oxygen in the presence of substrate and ADP) and 4 (consumption of oxygen after ADP has been consumed). Only the higher

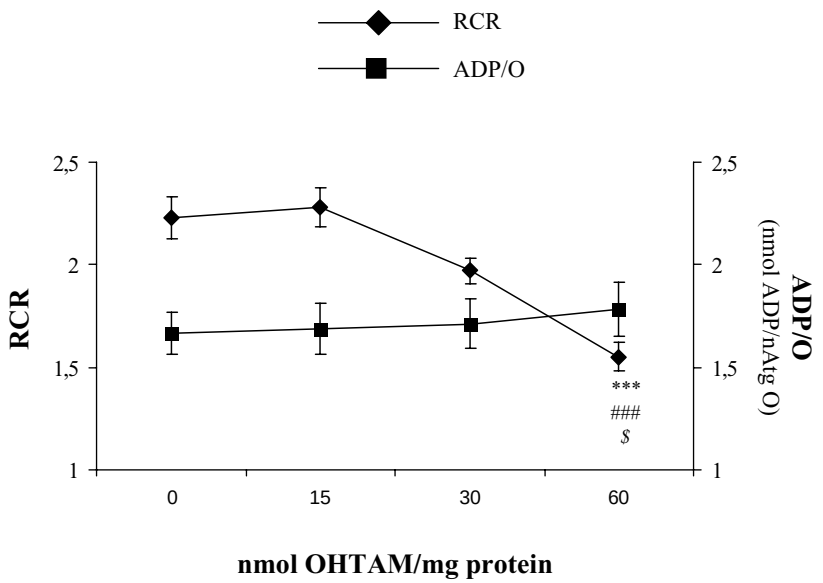

Fig. 1. Effect of OHTAM in respiratory control indexes (RCR and ADP/O ratio) of brain mitochondria. Freshly isolated brain mitochondria (0.6 mg) in 1 ml of the standard medium supplemented with 2 μM rotenone were energized with 5 mM succinate. Isolates were incubated with OHTAM for 2 min, at 30 °C, before mitochondria energization. Results are presented as mean ± S.E.M. of six independent experiments. (***) $P < 0.001$, when compared with mitochondria in control condition. (###) $P < 0.001$, when compared with mitochondria incubated with 15 nmol OHTAM /mg protein. ($) $P < 0.05$, when compared with mitochondria incubated with 30 nmol OHTAM/mg protein.

concentration of OHTAM (60 nmol/mg protein) induced a significant decrease on RCR when compared with control condition (30.49% ± 1.35) as well as when compared with 15 nmol OHTAM/mg protein (32.11% ± 1.16) and 30 nmol OHTAM/mg protein (21.40% ± 0.40) (Fig. 1).

ADP/O ratio, an indicator of oxidative phosphorylation efficiency, is expressed by the ratio between the amount of ADP added and the oxygen consumed during the state 3 of respiration. This parameter remained statistically unchanged in all the conditions studied (Fig. 1).

3.2. Effect of OHTAM in brain mitochondrial transmembrane potencial ($\Delta\Psi_m$), repolarization level, repolarization lag phase and ATP content

The mitochondrial transmembrane potential ($\Delta\Psi_m$) is fundamental for the phenomenon of oxidative phosphorylation, the conversion of ADP to ATP via ATP synthase. Mitochondrial respiratory chain pumps H^+ out of the mitochondrial matrix across the inner mitochondrial membrane. The H^+ gradient originates an electrochemical potential (Δp) resulting in a pH (ΔpH) and a voltage gradient ($\Delta\Psi_m$) across the mitochondrial inner membrane. As shown in Table 1, $\Delta\Psi_m$ remained unchanged in all the conditions tested. However, the repolarization level (capacity of mitochondria to reestablish the $\Delta\Psi_m$, after ADP phosphorylation) decreased significantly in the presence of 60 nmol OHTAM/mg protein when compared with all the other conditions tested (Table 1). Accordingly, the repolarization lag phase (time necessary for ADP phosphorylation) increased significantly in the presence of the highest OHTAM concentration tested.

Concerning the ATP content, we did not observe any significant alteration promoted by OHTAM. Although the presence of 60 nmol OHTAM/mg protein slowed the oxidative phosphorylation capacity of brain mitochondria

Table 1
Effect of OHTAM in the mitochondrial oxidative phosphorylation system (mitochondrial transmembrane potential ($\Delta\Psi_m$), repolarization level, repolarization lag phase and ATP content)

	Control	OHTAM (15 nmol/mg prot)	OHTAM (30 nmol/mg prot)	OHTAM (60 nmol/mg prot)
$\Delta\Psi_m$ (−mV)	186.6 ± 1.83	184.9 ± 2.36	183.5 ± 2.51	175.9 ± 3.06
Repolarization level (−mV)	157.5 ± 2.18	152.9 ± 1.69	151.0 ± 1.98	139.6 ± 2.29[***,##,$$]
Repolarization lag phase (min)	0.558 ± 0.05	0.588 ± 0.051	0.738 ± 0.036	0.922 ± 0.071[***,##]
ATP content (nmol/mg protein)	285.9 ± 17.75	279.5 ± 12.85	243.5 ± 13.33	272.4 ± 19.69

The oxidative phosphorylation parameters were evaluated using freshly isolated brain mitochondria (0.6 mg) in 1 ml of the reaction medium supplemented with 3 μM TPP^+ and 2 μM rotenone and energized with 5 mM succinate.

[***] $P < 0.001$, statistically significant when compared to control condition.

[##] $P < 0.01$, statistically significant when compared to 15 nmol OHTAM/mg protein.

[$$] $P < 0.01$, statistically significant when compared to 30 nmol OHTAM/mg protein. Data shown represent mean ± S.E.M. from six independent experiments.

(increase in repolarization lag phase and decrease in repolarization level) they were capable to phosphorylate all the ADP added to mitochondrial suspension.

3.3. Effect of OHTAM on lipid peroxidation: oxygen consumption and TBARS formation

The oxygen consumption and TBARS assay were used to determine the level of lipid peroxidation induced by ADP/Fe^{2+}. The maximal values of oxygen consumption (Fig. 2A) and TBARS formation (Fig. 2B), observed at 15 min of peroxidation, were 172.8 ± 23.79 nmol/mg protein and 22.75 ± 3.53 nmol TBARS/mg protein, respectively. Inhibition of oxygen consumption and TBARS formation was observed in the presence of all the concentrations of OHTAM tested (Fig. 2A and B). Although a concentration-dependent protection was observed, the presence of 7.5 nmol OHTAM/mg protein afforded a clear protection being the extension of lipid peroxidation not statistically different when compared with the other concentrations of OHTAM tested as well as when compared with the control condition.

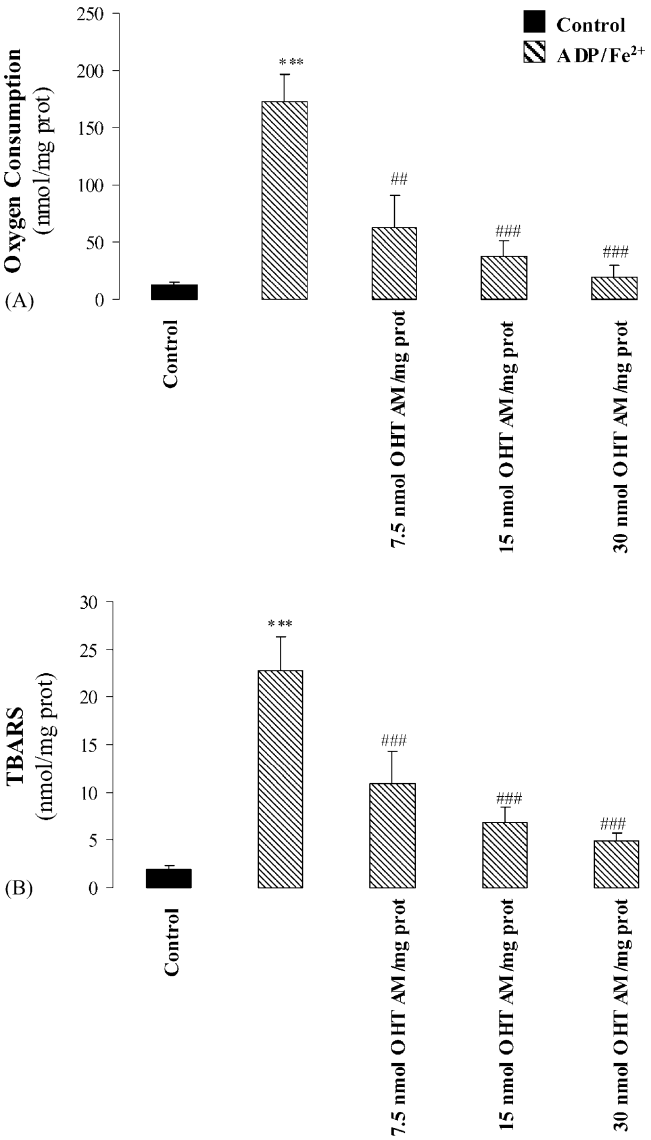

Fig. 2. Effect of OHTAM on lipid peroxidation: oxygen consumption (A) and TBARS formation (B). Freshly isolated brain mitochondria were incubated at 0.6 mg protein/ml under standard conditions as described in Section 2. Isolates were pre-incubated with OHTAM for 2 min, at 30 °C, before the addition of ADP-Fe^{2+}. Results are presented as mean ± S.E.M. of six independent experiments. (*) $P < 0.05$, when compared with mitochondria in control condition. (###) $P < 0.001$; (##) $P < 0.01$; (#) $P < 0.05$; when compared with mitochondria incubated with 30 μM Ca^{2+}.

3.4. Effect of OHTAM on the mitochondrial permeability transition pore (MPTP): $\Delta\Psi_m$ and calcium fluxes

The mitochondrial potential ($\Delta\Psi_m$) drop is a typical phenomenon that follows the induction of MPTP. In control conditions (Fig. 3A), the addition of 5 mM succinate produced an $\Delta\Psi_m$ of ≈185 mV (negative inside mitochondria), corresponding to the respiratory state 4. Then, the first pulse of 10 μM Ca^{2+} led to a rapid depolarization (decrease of $\Delta\Psi_m$) followed by a small repolarization (recover of $\Delta\Psi_m$). The depolarization was due to the entry of Ca^{2+} into the electronegative mitochondrial matrix, followed by the efflux of H^+ in an attempt to restore the $\Delta\Psi_m$. However, a second and third pulse of 10 μM Ca^{2+} led to a total depolarization of mitochondria. Mitochondria can tolerate some amount of Ca^{2+}, but ultimately their capacity to adapt to Ca^{2+} loads is overwhelmed and mitochondria depolarize completely due to a profound change in the inner membrane permeability. Since the mitochondrial collapse of $\Delta\Psi_m$, associated to Ca^{2+} overload, is related with MPTP opening, experiments were performed to further confirm the induction of the MPTP opening by studying the Ca^{2+} retention by energized mitochondria. Fig. 4 shows that isolated brain mitochondria incubated with 100 nmol Ca^{2+}/mg protein in the presence of 2 mM phosphate and energized with succinate, rapidly accumulate Ca^{2+} from the medium and, after a slow release of some of the accumulated Ca^{2+}, they were able to continue to accumulate Ca^{2+}, thus retaining part of the Ca^{2+} present in the medium. In the presence of 50 nmol Ca^{2+}/mg protein mitochondria retained a large portion of this cation, however in the presence of 150 nmol Ca^{2+}/mg protein mitochondria became unable to retain the accumulated Ca^{2+} due to MPTP opening (Fig. 4A).

These effects on calcium accumulation could be prevented in the presence of 0.85 μM CsA and 1 mM ADP plus 2 μg/ml oligomycin. Although both agents contributed for a higher capacity of mitochondria to accumulate Ca^{2+}, the protection exerted by ADP plus oligomycin was more effective than that exert by CsA (Figs. 3B, C and 4). The presence of 30 nmol OHTAM/mg protein (2 min preincubation) exerted some protection against mitochondrial membrane depolarization and calcium release, by increasing the capacity of mitochondria to acumulate Ca^{2+} (Figs. 3B and 4B). Additionally, the presence of 0.85 μM cyclosporin A (CsA) (specific inhibitor of MPTP)

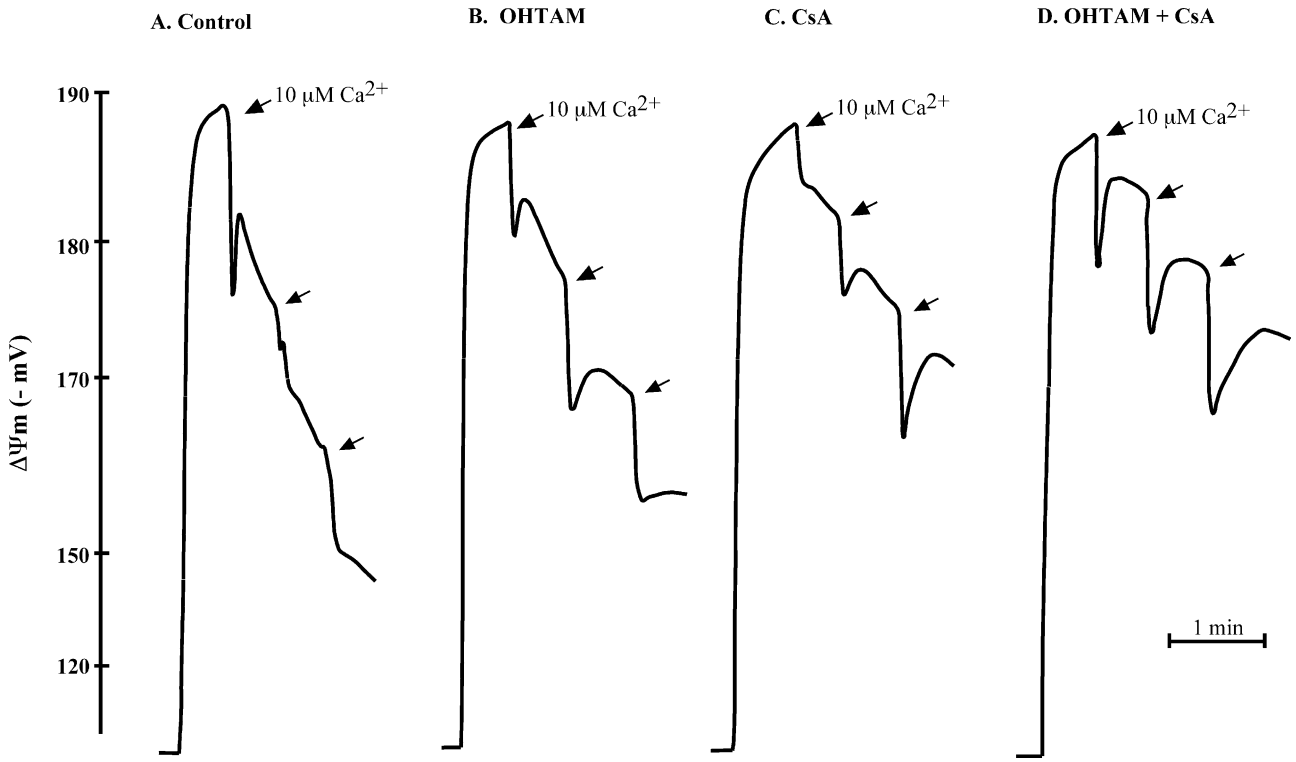

Fig. 3. Effect of OHTAM on mitochondrial transmembrane potential depolarization: permeability transition pore induction. Freshly isolated brain mitochondria (0.6 mg) in 1 ml of the standard medium supplemented with 3 μM TPP^+ and 2 μM rotenone were energized with 5 mM succinate. (A) Control condition; (B) 30 nmol OHTAM/mg protein; (C) 0.85 μM CsA; (D) 30 nmol OHTAM/mg protein plus 0.85 μM CsA. Isolates were pre-incubated with OHTAM and/or CsA for 2 min before mitochondria energization with succinate. Ca^{2+} (3 × 10 μM) was added 1.5 min after mitochondria energization. The traces are typical of six independent experiments.

added 2 min prior to Ca^{2+}, afforded a clear protection of mitochondria (Figs. 3C and 4). However, in the presence of both agents, 30 nmol OHTAM/mg protein and 0.85 μM CsA, the capacity of mitochondria to accumulate Ca^{2+} increased significantly being mitochondria more resistant to Ca^{2+} addition and, consequently, to MPTP induction (Figs. 3D and 4B).

3.5. Effect of OHTAM on the mitochondrial glutathione content

Theoretically, the opening of MPTP is stimulated by the alteration of GSH redox status (–SH → –SS–). We observed that Ca^{2+} induced a decrease in GSH (reduced glutathione) which was reversed in the presence of 30 nmol OHTAM/mg protein and/or CsA. However, GSSG (oxidized glutathione) did not change in all the conditions studied (Fig. 5). The decrease induced by Ca^{2+} in GSH was confirmed by the significant decrease of GSH/GSSG ratio being this decrease reverted by the presence of 30 nmol OHTAM /mg protein and/or CsA (Fig. 5).

4. Discussion

Several evidences from the literature indicate that mitochondria have a significant role to play not only in routine energy metabolism, but also in cell death [21]. Neurons are structures extremely susceptible to mitochondrial dysfunction since about 90% of the energy required by these cells is provided by mitochondria. In this order, the search of new compounds capable to protect mitochondria against toxic and/or stressful agents has become an important goal for many scientists working in this research area.

In this study the effect of OHTAM in brain mitochondrial function was evaluated. We observed that only higher concentrations of OHTAM (60 nmol/mg protein) promote a decrease in respiratory chain and oxidative phosphorylation efficiencies (Fig. 1, Table 1). In accordance, Cardoso et al. [36] observed that OHTAM decreases the respiratory coefficient and the ADP/O ratio of liver mitochondria in a concentration-dependent way. However, this depression of respiratory indexes was significant for concentrations above 40 nmol OHTAM/mg protein. Furthermore, they also observed that concentrations of OHTAM > 40 nmol/mg protein increases the lag phase of repolarization and decreases the rate of repolarization. Similarly, we observed a decrease on repolarization level and an increase on repolarization lag phase although these alterations occurred in the presence of higher OHTAM concentrations (60 nmol OHTAM/mg protein) (Table 1). The discrepancies in the concentrations of OHTAM in both studies are probably due to the different type of mitochondria used.

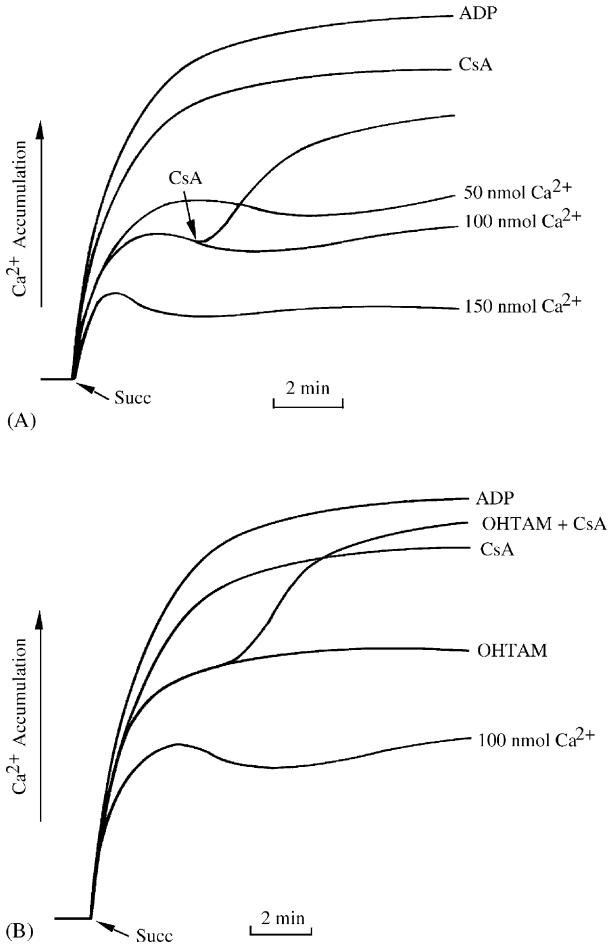

Fig. 4. Effect of OHTAM on calcium fluxes. Mitochondria were incubated at 0.8 mg protein/ml under standard conditions as described in Section 2. Standard medium was supplemented with Ca^{2+} 1 min before mitochondria energization with 5 mM succinate. 30 nmol OHTAM/mg protein, 0.85 μM CsA and 2 μg/ml oligomycin plus 1 mM ADP were added to the reaction medium 2 min prior Ca^{2+} addition. In CsA plus OHTAM condition, CsA was added after mitochondria energization. The uptake and release of sequestered Ca^{2+} by mitochondria were monitored as described in Section 2. The traces are typical of six independent experiments.

The existence of tissue-specific differences in mitochondria properties has been described [37,38].

Our results indicate that although a decrease in oxidative phosphorylation efficiency occurs in the presence of high concentrations of OHTAM, it do not interfere with ATP content of brain mitochondria (Table 1). Recently, Cardoso et al. [39] showed that only concentrations of OHTAM above 60 nmol/mg protein decrease ATP content of liver mitochondria.

However, OHTAM has shown to be a powerful antioxidant, protecting brain mitochondria against ADP/Fe^{2+}-induced lipid peroxidation. Our results show that OHTAM exert a potent antioxidant action since 7.5 nmol OHTAM/mg protein was sufficient to reduce oxygen consumption (Fig. 2A) and TBARS levels (Fig. 2B) to values similar to those found in control condition (basal values). It has been described that OHTAM is a scavenger of peroxyl radicals in several cells and systems. OHTAM inhibits lipid peroxidation in sarcoplasmic reticulum membranes [5] and Fe^{3+}-ascorbate-induced lipid peroxidation in rat liver microsomes [40]. Furthermore, the ability of TAM and OHTAM to inhibit Cu^{2+}-induced peroxidation of low-density lipoprotein has been suggested to contribute to the putative cardioprotective effects of these antiestrogens [41]. Raghvendra and coworkers [42] demonstrated that estrogen and tamoxifen metabolites protect smooth muscle cell membrane phospholipids against peroxidation via a non-estrogen receptor-dependent mechanism. A study from O'Neill et al. [43] showed that raloxifene (estrogen receptor modulator) at low concentrations (50 ng/ml) induces a significant neuroprotection against β-amyloid-, H_2O_2- and glutamate-induced toxicity. However, the same study showed that raloxifene exerts partial estrogen agonist action in the absence of 17 β-estradiol whereas in its presence raloxifene exerted a mixed estrogen agonist-antagonist effect. Similarly, Lei et al. [44] found that estrogen and raloxifene can influence glial-mediated inflammatory pathways and possibly protecting against age- and disease-related neuropathology. However, there are some evidences that TAM and OHTAM are associated with intracellular oxidative stress [45,46]. These discrepancies can be due to differences in the concentrations of TAM and OHTAM and/or experimental models used. Indeed, some animal studies indicate the existence of dose-related biphasic effects of estradiol [47,48]. Some effects are lost at higher concentrations [47,48], whereas others require high doses [49]. These dose-related effects could be taken as an advantage to possibly dissociate some activities in the target tissue. It is possible that a similar picture occurred with antiestrogen compounds.

Since previous studies [26] demonstrated the inhibitory effect of OHTAM on MPTP induction, we tested the effect of OHTAM on Ca^{2+}-induced MPTP of isolated brain mitochondria. MPTP causes a non-selective permeabilization of the inner mitochondrial membrane typically promoted by the accumulation of excessive quantities of Ca^{2+} ions and stimulated by a variety of compounds or conditions [24]. In this study, the classic inductor Ca^{2+} was used to induce MPTP opening (Figs. 3 and 4). Ca^{2+} uptake is concentration-dependent, with higher concentrations leading to a lower amount of Ca^{2+} being accumulated by mitochondria, indicating a continuous cycling of Ca^{2+} due to MPTP induction. The pore opening allows the equilibration of ions and respiratory substrate between cytosol and mitochondrial matrix leading to a reduction on $\Delta\Psi_m$ [50], which is linked to depletion of GSH [51]. Indeed, mitochondrial pore induction is intimately associated with GSH and protein thiol redox state [52]. Glutathione is the most abundant antioxidant in the cell. Regeneration of GSH from GSSG occurs in matrix. Continued regeneration of GSH requires NADPH, which in turn requires transhydrogenation of $NADP^+$ and NADH. In our study, we observed that Ca^{2+} induced a significant decrease in GSH/GSSG ratio due to a decrease in GSH

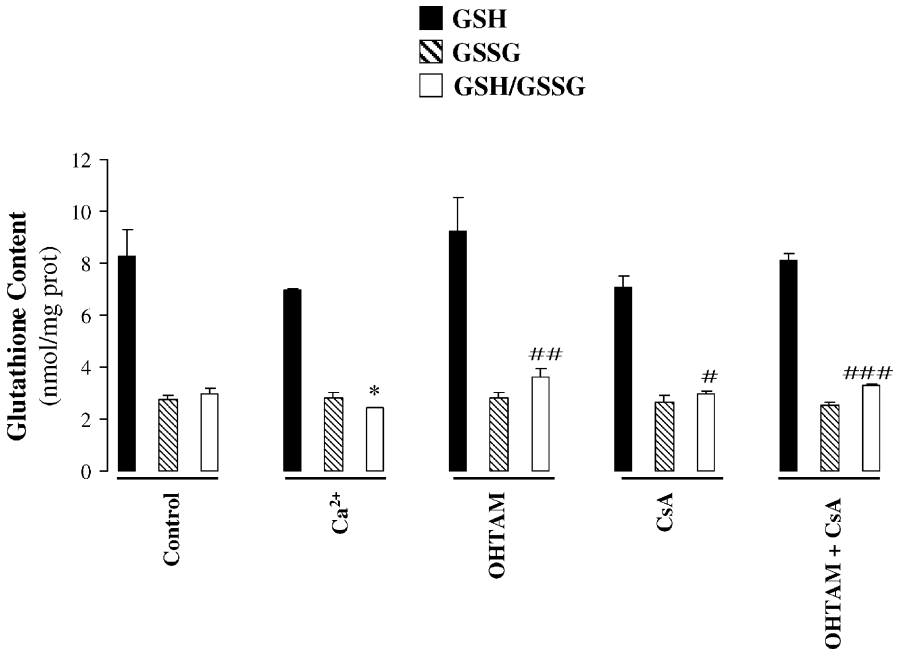

Fig. 5. Effect of OHTAM on mitochondrial GSH content. Freshly isolated brain mitochondria (0.6 mg) in 1 ml of the standard medium supplemented with 2 μM rotenone were energized with 5 mM succinate. Isolates were pre-incubated with OHTAM and/or CsA for 2 min, at 30 °C, before mitochondria energization. Ca^{2+} was added after mitochondria energization. Results are presented as mean ± S.E.M. of six independent experiments.

content since no alteration in GSSG content was observed (Fig. 5). Similarly, Custódio et al. [25] showed that the loss of matrix GSH from liver mitochondria is attributed to its release to the medium since no GSSG was detected in any of the reactions and since the total GSH (intra and extra-mitochondrial) did not change. So, the depletion of GSH was solely due to the efflux from the permeabilized mitochondria, which was prevented by cyclosporin A and hydroxytamoxifen (Fig. 5).

The presence of 30 nmol OHTAM/mg protein exerted some protection against Ca^{2+}-induced MPTP opening (Figs. 3B and 4B). Our results are supported by the observations of Morkuniene et al. [53] that OHTAM prevented calcium-induced loss of cytochrome *c* from heart mitochondria. In our study, CsA does not give a total protection against MPTP induction, however, it increases the capacity of brain mitochondria to accumulate Ca^{2+} (Figs. 3C and 4). Simpson et al. [54] observed that TAM exerted a CsA-like action on SH-SY5Y neuroblastoma mitochondria. Similarly, Cardoso et al. [26] observed that 20 μM OHTAM caused a total inhibition of Ca^{2+}-induced depolarization of succinate-energized liver mitochondria similar to that exerted by CsA. As discussed previously, due to tissue-specific differences in mitochondria pore properties [37], the ability of both agents, OHTAM and CsA, to protect against MPTP induction differs significantly between different types of mitochondria. The effect of CsA on the ability of brain mitochondria to accumulate Ca^{2+} was only moderate when compared with the effects described for CsA on muscle or liver mitochondria [38,55]. Similar effects of CsA were also reported on cell lines [56] and neuronal primary cultures [57]. Another study performed in a neuronal primary culture showed that mitochondrial depolarization induced by glutamate was partially inhibited by TAM [27]. Interestingly, 30 nmol OHTAM/mg protein plus CsA exerted a higher protection against MPTP opening (Fig. 3D and 4B) when compared with the protection exerted by each agent alone (Fig. 3B, C and 4). These results indicate that the effects of both agents are not additive, suggesting that they act at different places on mitochondrial pore complex. The most plausible hypothesis concerning MPTP inhibition by CsA is that cyclophilin D binding to the matrix side of the MPTP favors its opening and that CsA indirectly causes pore closure through unbinding of cyclophilin D after formation of the CsA-cyclophilin complex [58]. In turn, MPTP inhibition by OHTAM may be due to its antioxidant properties [5]. This hypothesis is supported by our results that show that OHTAM exerts potent antioxidant effects against lipid peroxidation (Fig. 2A and B).

Our results support the idea that the neuroprotective role of OHTAM is primarily due to its antioxidant-like actions. However, we cannot rule out its capacity in MPTP inhibition, especially in the presence of CsA, exacerbating its inhibitory effect in MPTP induction. In this order, new therapeutic strategies can be designed for the treatment of neurodegenerative disorders where mitochondrial dysfunction, resulting among other factors from lipid peroxidation and MPTP induction, may have a determinant role in neuronal cell degeneration and death.

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