Tetrandrine concentrations not affecting oxidative phosphorylation protect rat liver mitochondria from oxidative stress

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Abstract

The effects of tetrandrine (6,6', 7,12-tetramethoxy-2, 2'-dimethyl-berbaman) on the mitochondrial function were assessed on oxidative stress, mitochondrial permeability transition (MPT), and bioenergetics of rat liver mitochondria. At concentrations lower than 100 nmol/mg protein, tetrandrine decreased the hydrogen peroxide formation, the extent of lipid peroxidation, the susceptibility to Ca2+-induced opening of MPT pore, and inhibited the inner membrane anion channel activity, not significantly affecting the mitochondrial bioenergetics. High tetrandrine concentrations (100–300 nmol/mg protein) stimulated succinate-dependent state 4 respiration, while some inhibition was observed for state 3 and p-trifluoromethoxyphenylhydrazone-uncoupled respirations. The respiratory control ratio and the transmembrane potential were depressed but the adenosine diphosphate to oxygen (ADP/O) ratio was less affected. A slight increase of the inner mitochondrial membrane permeability to H+ and K+ by tetrandrine was also observed. It was concluded that low concentrations of tetrandrine afford protection against liver mitochondria injury promoted by oxidative-stress events, such as hydrogen peroxide production, lipid peroxidation, and induction of MPT. Conversely, high tetrandrine concentrations revealed toxicological effects expressed by interference with mitochondrial bioenergetics, as a consequence of some inner membrane permeability to H+ and K+ and inhibition of the electron flux in the respiratory chain. The direct immediate protective role of tetrandrine against mitochondrial oxidative stress may be relevant to clarify the mechanisms responsible for its multiple pharmacological actions.

Keywords: Bisbenzylisoquinoline alkaloid; Cancer; Mitochondrial bioenergetics; Permeability transition pore; Tetrandrine

1. Introduction

Mitochondria, whose main function is ATP synthesis by oxidative phosphorylation, have many other roles including the modulation of intracellular Ca2+ concentration and the regulation of apoptotic cell death (Lee and Wei, 2000; Murphy and Smith, 2000; Pedersen, 2000). Mitochondria are also considered to be, under normal and pathological conditions, the major endogenous source of reactive oxygen species (ROS), and perhaps of oxidative stress in general (Lee and Wei, 2000; Murphy and Smith, 2000; Pedersen, 2000).

Oxidative damage to mitochondria leads to a decline in the efficiency of oxidative phosphorylation and, in conditions of Ca2+ loading, it induces mitochondrial permeability transition (MPT), release of cytochrome c and other factors able to trigger apoptosis (Kowalski and Vercesi, 1999; Kowalski et al., 2001).

Tetrandrine (6,6', 7,12-tetramethoxy-2, 2'-dimethyl-berbaman), a bisbenzylisoquinoline alkaloid (Fig. 1) isolated from the dried root of the Chinese herb Stephania tetrandra (S. Moore), has been used as an effective anti-hypertensive and anti-arrhythmic agent in modern China (Wang et al., 2004). Tetrandrine blocks voltage-gated Ca2+ channels, large-conductance Ca2+-activated K+ (BK) channels, and intracellular Ca2+ pumps (Wang...
2. Materials and methods

2.1. Animals

Male Wistar rats (250–350 g), housed at 22 ± 2 °C under artificial light for 12-h light/dark cycle and with access to water and food ad libitum, were used throughout the experiments. The experiments reported here were carried out in accordance with the National Requirements for Vertebrate Animal Research and in accordance with the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes.

2.2. Isolation of rat liver mitochondria

Rat liver mitochondria were isolated from male Wistar rats (6 weeks) by differential centrifugation according to conventional methods (Gazotti et al., 1979). After washing, the pellet was gently resuspended in the washing medium at a protein concentration of about 50 mg/ml. Protein content was determined by the biuret method (Gornall et al., 1949), using bovine serum albumin as a standard.

2.3. Hydrogen peroxide generation

Hydrogen peroxide (H$_2$O$_2$) generation was measured fluorimetrically using a modification of the method previously described (Barja, 2002). In brief, mitochondria (0.375 mg) were incubated at 30 °C in 1.5 ml of phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 145 mM KCl, 30 mM Hepes, 0.1 mM homovalinic acid, and 6 U/ml horseradish peroxidase. The reactions were started by adding succinate (10 mM) and stopped at 15 min with 0.5 ml of cold 2 M glycine–NaOH (pH 12) containing 25 mM EDTA. The mitochondrial suspensions were centrifuged at 850g for 10 min. The fluorescence of supernatants was measured at 312 nm as excitation and 420 nm as emission wavelengths. The peroxide generation was calculated using a standard curve of H$_2$O$_2$. The standards and samples were incubated under the same conditions.

2.4. Lipid peroxidation

The extent of lipid peroxidation was evaluated by oxygen consumption using a Clark-type electrode, in a closed glass chamber equipped with magnetic stirring, thermostated at 30 °C. Mitochondria (1.5 mg) were pre-incubated for 3 min in 1.5 ml of a medium containing 175 mM KCl, 10 mM Tris–Cl (pH 7.4), supplemented with 3 μM rotenone (in the presence or absence of tetrandrine) to avoid mitochondrial respiration induced by endogenous respiratory substrates. Iron solution was prepared immediately before use and protected from light. The changes in O$_2$ tension were recorded in a potentiometric chart record and oxygen consumption calculated assuming an oxygen concentration of 232 nmol O$_2$/ml. Membrane lipid peroxidation was initiated by adding 1 mM adenosine diphosphate (ADP)/0.1 mM Fe$^{2+}$ as oxidizing agents. Controls, in the absence of ADP/Fe$^{2+}$, were performed under the same conditions.

Lipid peroxidation was also determined by measuring thiobarbituric acid reactive substances (TBARS), using the thiobarbituric acid assay (Ernster and Nordenbrand, 1967). Aliquots of mitochondrial suspensions (0.5 ml each),
removed 10 min after the addition of ADP/Fe^{2+}, were added to 0.5 ml of ice-cold 40% trichloroacetic acid. Then, 2 ml of 0.67% of aqueous thiobarbituric acid containing 0.01% of 2,6-di-tert-butyl-p-cresol was added. The mixtures were heated at 90 °C for 15 min, then cooled in ice for 10 min, and centrifuged at 850g for 10 min. The supernatant fractions were collected and lipid peroxidation was estimated spectrophotometrically at 530 nm. The amount of TBARS formed was calculated using a molar extinction coefficient of 1.56 × 10^5/mol/cm and expressed as nmol TBARS/mg protein (Buege and Aust, 1978).

2.5. Extramitochondrial calcium movements

Extramitochondrial-free Ca^{2+} was measured with the hexapotassium salt of the fluorescence calcium, sensitive probe Calcium Green 5-N (Rajdev and Reynolds, 1993). Mitochondria (0.25 mg) were suspended in 2 ml of buffer containing 200 mM sucrose, 10 mM Hepes–Tris (pH 7.4), 1 mM KH_2PO_4 and 10 μM EGTA, 2 μM rotenone, 1 μg oligomycin, and 80 nmol Ca^{2+}/mg protein. Free Ca^{2+} was monitored with 100 mM Calcium Green 5-N. Fluorescence was continuously recorded in a water-jacked cuvette holder at 30 °C, using a Perkin–Elmer LS-50B fluorescence spectrometer with excitation and emission wavelengths of 506 and 531 nm, respectively. Calcium uptake by mitochondria was initiated by addition of 5 mM succinate. The minimum fluorescence of mitochondrial suspensions, after succinate addition, corresponds to maximum calcium uptake by mitochondria.

2.6. Measurement of respiratory activities

Oxygen consumption was monitored polarographically with a Clark-type electrode, in a closed glass chamber equipped with magnetic stirring, thermostated at 30 °C. Mitochondria (1.5 mg protein) were incubated in 1.5 ml of medium containing 250 mM sucrose, 10 mM Hepes (pH 7.2), 20 mM KCl, 5 mM KH_2PO_4, and 2 mM MgCl_2, supplemented with 2 μM rotenone and 3 μM TPP^+, at 30 °C, before energization with 10 mM succinate. No correction was made for the “passive” binding of TPP^+ to the mitochondria membranes because the purpose of the experiments was to show relative changes in potential rather than absolute values. As a consequence, we can anticipate some overestimation for the ΔΨ values. Tetrandrine did not affect TPP^+ binding to mitochondria membranes or the electrode response.

2.7. Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential (ΔΨ) was measured indirectly based on the activity of the lipophilic tetraphenylphosphonium (TPP^+) using a TPP^+-selective electrode, as previously described (Kamo et al., 1979). Mitochondria (1.5 mg protein) were incubated for 3 min in 1.5 ml of medium containing 250 mM sucrose, 10 mM Hepes (pH 7.2), 20 mM KCl, 5 mM KH_2PO_4, and 2 mM MgCl_2, supplemented with 2 μM rotenone and 3 μM TPP^+, at 30 °C, before energization with 10 mM succinate. Mitochondria (1.5 mg protein) were incubated in 1.5 ml of medium containing 250 mM sucrose, 10 mM Hepes–Tris (pH 7.4), 1 mM KH_2PO_4, 10 μM EGTA, 2 μM rotenone, and 2 mM MgCl_2, supplemented with 2 μM rotenone. Assays were performed in the presence of nigericin (1 nmol/mg protein), and valinomycin (1 mM rotenone). Assays were performed in the presence of nigericin (1 nmol/mg protein), and valinomycin (1 μg/mg protein). O_2 consumption was calculated considering that the saturation oxygen concentration was 232 nmol O_2/ml. Control values are expressed in nmol O_2/mg protein/min. The respiratory control ratio (RCR) and ADP to oxygen ratio (ADP/O) were calculated according with a previously described method (Chance and Williams, 1956).

2.8. Mitochondrial swelling

Mitochondrial osmotic volume changes were measured by the apparent absorbance changes at 520 nm with a suitable spectrophotometer-recorder set up. Mitochondrial swelling methods were used to detect H^+ and K^+ mitochondrial inner membrane permeabilization (Vicente et al., 1998), activity of mitochondrial inner membrane anion channel (IMAC) (Beavis et al., 1985), and Ca^{2+}-induced MPT (Costantini et al., 1996). The reactions were carried out at 30 °C, with 1 mg mitochondrial protein in 2.5 ml of the required media, as described below.

Mitochondrial inner membrane permeabilization to H^+ was detected in K-acetate medium [135 mM K-acetate, 5 mM Hepes (pH 7.1), 0.1 mM EGTA, and 0.2 mM EDTA] supplemented with 2 μM rotenone. All assays were performed in the presence of 1 μg/ml valinomycin to permeabilize to K^+. A control assay was performed in the presence of 1 μM FCCP for total permeabilization to H^+. Other assays were performed in the presence of tetrandrine instead of FCCP.

To detect K^+ mitochondrial inner membrane permeabilization, we used K-nitrate medium [135 mM KNO_3, 5 mM Hepes (pH 7.1), 0.1 mM EGTA, and 0.2 mM EDTA] supplemented with 2 μM rotenone. Some assays were performed in the presence of 1 μg/ml valinomycin for total permeabilization to K^+. Other assays were performed in the presence of valinomycin instead of FCCP.

Detection of IMAC activity used KCl medium [135 mM KCl, 5 mM Hepes (pH 7.4), 0.1 mM EGTA, and 0.2 mM EDTA] supplemented with 2 μM rotenone. Assays were performed in the presence of nigericin (1 nmol/mg protein), and valinomycin (1 μg/mg protein), in the absence or presence of tetrandrine. Control assays, in the absence of valinomycin or tetrandrine, were also performed. The IC_{50} value, concentration causing 50% of maximal inhibition for IMAC, was determined considering initial rates of swelling, as previously described (Zernig et al., 1990).

To detect Ca^{2+}-induced MPT, we used a medium containing 200 mM sucrose, 10 mM Hepes–Tris (pH 7.4), 1 mM KH_2PO_4, 10 μM EGTA, 2 μM rotenone, and 2 mM MgCl_2, supplemented with 2 μM rotenone. Some assays were performed in the presence of nigericin (1 nmol/mg protein), and valinomycin (1 μg/mg protein), in the absence or presence of tetrandrine. Control assays, in the absence of valinomycin or tetrandrine, were also performed. The IC_{50} value, concentration causing 50% of maximal inhibition for IMAC, was determined considering initial rates of swelling, as previously described (Zernig et al., 1990).
1 μg/ml of oligomycin, in the absence and presence of
tetrandrine. Mitochondria were energized with 5 mM succi-
cinate and 80 nmol Ca^{2+}/mg protein was added to induce
MPT. Control assays in the absence of Ca^{2+}, and in pres-
ence of Ca^{2+} plus 1 μM cyclosporin A (CsA) were also
performed.

2.9. Statistical analysis

All the experiments were performed using four indepen-
dent experiments with different mitochondrial prepara-
tions. The values are expressed as mean ± SEM Means
were compared using ANOVA. Statistical significance
was set at \( p < 0.05 \).

2.10. Chemicals

All chemicals were obtained from Sigma Chemical
Company (St Louis, MO, USA) except for tetrandrine,
which was purchased from Aldrich (Madrid, Spain). Tet-
randrine was dissolved in absolute dimethyl formamide
(DMF) and diluted with ethanol. Pure solutions of DMF
and ethanol (when necessary) were added for controls at
the highest volume used in tetrandrine solutions [1% (v/v)
of the experiments final volume], having no effects on the
measured activities.

3. Results

3.1. Effects of tetrandrine on mitochondrial oxidative stress

The effects of tetrandrine on ROS production and oxi-
dative damage were assessed detecting H\(_2\)O\(_2\) generated by
mitochondria (Fig. 2) and mitochondrial membrane lipid
peroxidation induced by the pro-oxidant pair ADP/Fe\(^{2+}\)
(Fig. 3), respectively. In the absence of tetrandrine, the
H\(_2\)O\(_2\) generated by succinate-respiring mitochondria signif-
ically increased as compared with non-respiring mito-
chondria (Fig. 2). Treatment of mitochondria with
antimycin A, an inhibitor of complex III, maximally stim-
ulated the H\(_2\)O\(_2\) produced by succinate-respiring mito-
chondria. This effect was strongly reduced by catalase to
levels (more than 80% inhibition) below those observed

![Fig. 2. Effect of tetrandrine on hydrogen peroxide production by rat liver
mitochondria. Mitochondria were incubated under standard conditions as
described in Section 2. The results correspond to the mean ± SEM of four
experiments obtained from different mitochondrial preparations. \(* * * \ p < 0.001\) and \(* * \ p < 0.01\), when compared with basal production of
H\(_2\)O\(_2\) [in the absence of succinate (black bar)]; \( \$ \ p < 0.01\), when compared
with control conditions [with succinate (dashed bars)]; \( \& \ p < 0.05\), when
compared with maximal production of H\(_2\)O\(_2\) [in the presence of succinate
plus 10 μM antimycin A (dotted bars)]; \( \& \ p < 0.05\) and \( \& \ p < 0.05\), when
compared with the respective controls [without tetrandrine (Tet)]. Assays
in the presence of succinate plus 10 μM antimycin A plus 643 U/ml catalase (white bar).]

![Fig. 3. Effect of tetrandrine on membrane lipid peroxidation of rat liver mitochondria induced by the pro-oxidant pair ADP/Fe\(^{2+}\). Lipid peroxidation was
evaluated by oxygen consumption (A) and TBARS assay (B). The traces in (A) represent typical direct recordings representative of four experiments
obtained from different mitochondrial preparations; –ADP/Fe\(^{2+}\), controls in the absence of ADP/Fe\(^{2+}\). The data in (B) correspond to the mean ± SEM of
four independent experiments. \( \& \ p < 0.05\); \( ** \ p < 0.01\); \( *** \ p < 0.001\); \( **** \ p < 0.0001\) as compared with mitochondria submitted to ADP/Fe\(^{2+}\) treatment
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M.A.S. Fernandes et al. / Mitochondrion 6 (2006) 176–185
in basal conditions (mitochondria in the absence of succinate), considering this as H$_2$O$_2$ assay control. Tetrandrine concentrations up to 80 nmol/mg protein significantly reduced H$_2$O$_2$ generation by succinate-respiring mitochondria either in the absence or presence of antimycin A. A maximal decrease of H$_2$O$_2$ generation (of about 50%) was observed at 80 nmol tetrandrine/mg protein for both conditions.

Fig. 3 shows the effect of tetrandrine on lipid peroxidation of rat liver mitochondria. This effect was evaluated by measuring oxygen consumption (Fig. 3A) and TBARS formation (Fig. 3B) due to membrane peroxidation induced by the pro-oxidant pair ADP/Fe$^{2+}$. In the absence of tetrandrine and after the addition of the pro-oxidant pair, it is possible to distinguish a two-phase kinetics in oxygen consumption: an initial lag phase, slow oxygen consumption, lasting about 1 min, is followed by a rapid oxygen consumption phase. The lag phase is probably related with the time required for the generation of a sufficient amount of the perferryl ion complex (ADP–Fe$^{2+}$–O$_2$ $\rightleftharpoons$ ADP–Fe$^{3+}$–O$_2$) which has been suggested to be responsible for the initiation of lipid peroxidation. The rapid oxygen consumption phase is probably due to the oxidation of the polyunsaturated fatty acid acyl chain of membrane phospholipids by ROS and, consequently, to the propagation phase of lipid peroxidation (Sassa et al., 1990). Tetrandrine concentrations up to 100 nmol/mg protein enlarge the lag phase of slow oxygen consumption before oxygen uptake burst induced by the complex ADP/Fe$^{2+}$, and decrease the consequent rate of the rapid oxygen consumption phase (Fig. 3A). This suggests that tetrandrine inhibits both the initiation and the propagation of lipid peroxidation of mitochondrial membranes with total inhibition of lipid peroxidation at 80 nmol/mg protein. These results agree with the quantitative evaluation of TBARS formation performed to confirm the clear protective effect of tetrandrine observed by oxygen consumption. Fig. 3B shows that the kinetics of TBARS formation induced by ADP/Fe$^{2+}$ is similar to that observed for oxygen consumption. The same range of tetrandrine concentrations used to inhibit oxygen consumption inhibited TBARS formation. TBARS formation in the absence of ADP/Fe$^{2+}$ is negligible (results not shown).

### 3.2. Effect of tetrandrine on Ca$^{2+}$-induced MPT

The effect of tetrandrine on Ca$^{2+}$-induced MPT was evaluated by passive osmotic swelling (Fig. 4A), ΔΨ dissipation (Fig. 4B), and Ca$^{2+}$ release (Fig. 4C). It is shown, in Fig. 4A, that swelling occurs in Ca$^{2+}$-loaded, succinate-energized rat liver mitochondria. CsA, a known MPT inhibitor (Broekemeier et al., 1989), totally blocks this effect, showing that swelling has been induced by MPT. Treatment of mitochondria with tetrandrine concentrations up to 20 nmol/mg protein, prior to adding succinate and Ca$^{2+}$, enlarged the lag time required for mitochondria to induce Ca$^{2+}$-dependent MPT. With the TPP$^+$-electrode, this inhibitory effect of tetrandrine was also investigated on Ca$^{2+}$-induced dissipation of ΔΨ associated with MPT induction (Fig. 4B). In the absence of tetrandrine, addition of Ca$^{2+}$ (80 nmol/mg protein) to succinate-energized mitochondria caused a transient depolarization followed by fast repolarization, after Ca$^{2+}$ accumulation. This repolarization is sustained for a short lag time before irreversible ΔΨ dissipation. Similarly to CsA, tetrandrine concentrations up to 20 nmol/mg protein, prior to succinate addition and Ca$^{2+}$, enlarged the lag time required for Ca$^{2+}$-induced irreversible depolarization of ΔΨ. This means that, in the presence of tetrandrine, the Ca$^{2+}$ uptake causes ΔΨ dissipation.
pation but, after Ca\(^{2+}\) accumulation, mitochondria develop sustained ΔΨ, demonstrating the inhibitory effect of tetrandrine on Ca\(^{2+}\)-induced MPT. Fluorescent calcium-sensitive probe Calcium Green 5-N (Fig. 4C), like TPP\(^{+}\)-electrode and swelling assays, also confirmed the results of inhibition of MPT induction by tetrandrine, reinforcing the conclusion that tetrandrine protects mitochondria from Ca\(^{2+}\)-induced MPT, not affecting the ability of mitochondria to accumulate Ca\(^{2+}\).

3.3. Effects of tetrandrine on the IMAC activity

The effect of tetrandrine on IMAC activity was evaluated by passive osmotic swelling of non-respiring rat liver mitochondria suspended in KCl medium (Fig. 5). In the presence of the ionophore A23187, mitochondrial matrix Mg\(^{2+}\) is depleted in exchange for H\(^{+}\), strongly increasing Cl\(^{-}\)/C\(_{0}\) permeability via IMAC. So, in the presence of valinomycin to permeabilize K\(^{+}\), plus nigericin to induce K\(^{+}\)/H\(^{+}\) antiport, A23187 strongly stimulates swelling of non-respiring mitochondria suspended in KCl medium. This swelling is increasingly prevented by the presence of tetrandrine concentrations up to 62.5 nmol/mg protein, indicating that IMAC-dependent Cl\(^{-}\) transport has been inhibited. The IC\(_{50}\) value for IMAC inhibition by tetrandrine was 12.5 ± 2 nmol/mg protein.

3.4. Effects of tetrandrine on mitochondrial bioenergetics

The effects of tetrandrine on succinate-supported respiratory rates (state 4, state 3, and FCCP-uncoupled respiration) and respiratory indices RCR and ADP/O ratio of rat liver mitochondria are significant at concentrations higher than 100 nmol/mg protein. As observed in Fig. 6, state 4 respiration was maximally stimulated at 300 nmol/mg protein (40%). At this concentration, state 3 respiration was depressed (30%) at a higher level than uncoupled respiration (15%) (Fig. 6A). Tetrandrine, at the concentration range up to 300 nmol/mg protein, significantly depressed RCR (from about 6 to less than 3), but the ADP/O ratio was only slightly decreased (from about 2 to 1.7) (Fig. 6B), suggesting a higher influence on the membrane permeability than on some specific oxidative phosphorylation interveners. These results are confirmed by Fig. 7, where tetrandrine dissipates ΔΨ induced by succinate-dependent respiration, consequently decreasing oxidative phosphorylation efficiency.

As a putative mechanism to explain the results described above, mitochondrial inner membrane permeabilizations to H\(^{+}\) and K\(^{+}\) by tetrandrine were evaluated by swelling of non-respiring mitochondria suspended in potassium acetate and potassium nitrate media, respectively (Fig. 8). Protonated acetate can cross the mitochondrial inner

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Fig. 5. Effect of tetrandrine on the mitochondrial inner membrane anion channel (IMAC). Assays were performed as described in Section 2, in the absence or presence of tetrandrine (Tet). Nig, 1 nmol/mg protein nigericin; Val, 1 nmol/mg protein valinomycin; A23187, 10 nmol/mg protein, 4 min after valinomycin; -Val, assay performed in the absence of valinomycin and tetrandrine. The traces are typical recordings representative of four experiments obtained from different mitochondrial preparations.

Fig. 6. Effect of tetrandrine on succinate-supported respiration of rat liver mitochondria. (A) O\(_{2}\) consumption in state 4 (open squares), state 3 (open circles), and uncoupled respiration (open triangles). (B) Respiratory indices RCR (closed circles), and ADP/O (closed squares). Control values expressed in nmol O\(_{2}\)/mg protein. min: state 4 = 8.9 ± 0.82; state 3 = 55.06 ± 0.45; uncoupled respiration = 80.22 ± 2.25. The results correspond to the mean ± SEM of four experiments obtained from different mitochondrial preparations. *p < 0.05; **p < 0.01, when compared with the control (without tetrandrine).
membrane and then, in the mitochondrial matrix, dissociate to the acetate anion and H⁺, producing a proton gradient. A valinomycin-dependent swelling only occurs if the proton gradient is dissipated. Tetrandrine concentrations up to 300 nmol/mg protein lead to a low rate of valinomycin-dependent mitochondrial swelling, indicating small proton conductance action through the mitochondrial inner membrane (15% of the maximum estimated with FCCP) (Fig. 8A). Mitochondrial inner membrane is permeable to nitrate (NO₃⁻/CO₃²⁻), but optimal swelling in potassium nitrate medium (KNO₃) is observed only in conditions of K⁺permeabilization. Maximum rate of swelling is observed by adding valinomycin to provide K⁺entry. Addition of tetrandrine concentrations up to 300 nmol/mg protein, instead of valinomycin, only induced a small swelling rate (17%), indicating that the K⁺ conductance through the inner mitochondrial membrane was only slightly affected by the highest tested concentration of tetrandrine (Fig. 8B).

4. Discussion

Tetrandrine protects several types of cells from oxidative stress by acting as a ROS scavenger, namely neutrophils (Seow et al., 1988; Matsuno et al., 1990; Castranova, 1994; Hui et al., 1996; Shen et al., 1999a,b, 2001), cerebellar granule neurons (Koh et al., 2003), erythrocytes (Sekiya et al., 2005), and tissues from heart (Ren et al., 1995) and liver (Liu et al., 2004). Tetrandrine was shown to induce depression of H₂O₂ production and inhibition of O₂⁻ radical generation (Seow et al., 1988), and also to efficiently react with .OH and to scavenge O₂⁻ radical (Shi et al., 1995; Cao, 1996; Ye et al., 2000). It has been demonstrated, using the Fenton reaction for .OH production and xanthine/xanthine oxidase for O₂⁻ production, that tetrandrine is an antioxidant comparable with ascorbate, glutathione, and cysteine (Cao, 1996; Ye et al., 2000). In quite agreement with these authors, we showed that, using liver mitochondrial fractions, tetrandrine directly protected from oxidative stress at concentrations not affecting oxidative phosphorylation. Both depression of H₂O₂ (Fig. 2) and inhibition of membrane lipid peroxidation (Fig. 3) detected the protective effect of tetrandrine from mitochondrial oxidative stress. Depression of H₂O₂ production by tetrandrine can be a result of its ability to scavenge O₂⁻, a ROS generated by the mitochondrial respiratory chain activity, which is then dismutated into H₂O₂ by the mitochondrial enzyme superoxide dismutase and/or it can be due to the ability of tetrandrine to induce mild uncoupling, resulting in slight ΔΨ dissipation (Cadenas and Davies, 2000; Lenaz, 2001; Ricci et al., 2003; Cadenas, 2004; Brookes, 2005; Jezek and Hlavatá, 2005). Indeed, low concentrations of tetrandrine (50 and 100 nmol/mg protein) induce slight but

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Fig. 7. Effect of tetrandrine on succinate-dependent transmembrane potential (ΔΨ) and phosphorylation of rat liver mitochondria. ADP, addition of 100 nmol ADP; Suc, 10 mM succinate; Tet, tetrandrine. The traces are typical recordings representative of four experiments obtained from different mitochondrial preparations. The lower table shows the mean ± SEM of membrane potential at the different indicated situations. *p < 0.05, values statistically different from control (in the absence of tetrandrine).
significant dissipation of $\Delta \Psi$ (Fig. 7). The inhibitory action of tetrandrine on membrane lipid peroxidation can be a result of its ability to prevent ROS production derived from ADP/Fe$^{2+}$. In so doing, tetrandrine avoids formation of the perferryl ion complex, ADP–Fe$^{3+}$–O$_2$ and, consequently, the oxidation of the polyunsaturated fatty acid acyl chain of membrane phospholipids, a mechanism suggested to be responsible for lipid peroxidation (Sassa et al., 1990).

IMAC is a non-selective anion channel that carries a wide variety of anions and it is regulated by Mg$^{2+}$ and H$^+$ matrix concentrations (Beavis, 1992), and by oxidative stress (Aon et al., 2003). IMAC is believed to be involved in mitochondrial volume homeostasis (Beavis, 1992), in the efflux of the O$_2^-$ anion from mitochondrion during ischemia preconditioning (Vanden Hoek et al., 1998), and in synchronized oscillations of mitochondrial membrane potential from isolated cardiac myocytes (O’Rourke, 2000; Aon et al., 2003).

It has been demonstrated that IMAC blockers avoid O$_2^-$ efflux from respiring mitochondria, protecting cells from degradative effects of ROS (Aon et al., 2003). Low concentrations of tetrandrine, interfering with the Cl$^-$ movement across the mitochondrial membrane (Fig. 5), strongly inhibit IMAC (IC$_{50}$ = 12.5 ± 2 nmol/mg protein), indicating that the compound is a potent IMAC blocker. We suggest that tetrandrine, acting as IMAC blocker (Fig. 5) and also as O$_2^-$ radical scavenger (Shi et al., 1995; Cao, 1996; Ye et al., 2000), can protect cells from oxidative stress. Furthermore, the interaction of tetrandrine with IMAC, admits interference with Cl$^-$ movements. Indeed, others showed that tetrandrine inhibited the Ca$^{2+}$-activated Cl$^-$ channel (a volume-regulated Cl$^-$ channel) inhibiting cell proliferation in cultured human umbilical vein endothelial cells (Fang et al., 2004). This suggests that interaction of the compound with anion channels can be relevant for its pharmacological activity as an anti-cancer drug.

Confirming tetrandrine protective action from oxidative stress, we observed that very low concentrations ($\leq$20 nmol/mg protein) protected rat liver mitochondria...
against Ca\(^{2+}\)-induced MPT, as revealed by different methods (Fig. 4). The ability of mitochondria to accumulate Ca\(^{2+}\) was not affected, showing no interference with Ca\(^{2+}\) transport. In fact, tetrandrine-induced repolarization and Ca\(^{2+}\) re-uptake by mitochondria after the onset of MPT (results not shown). This suggests that tetrandrine action, avoiding ROS production, inhibits induction of MPT, not interfering with Ca\(^{2+}\) uptake machinery. A critical factor for induction of MPT is the oxidation of protein thiol groups of the pore complex, creating diethyl cross-links (Costantini et al., 1996; Halestrap et al., 1997; McStay et al., 2002). As tetrandrine protects rat liver mitochondria from ROS production and membrane lipid peroxidation (Figs. 2 and 3), the most plausible hypothesis to explain MPT inhibition is concerned with the prevention of oxidative stress-induced thiol-bridge formation.

Concerning the discrepancy between our “in vitro” results, revealing protection of rat liver mitochondria against Ca\(^{2+}\)-induced MPT, and the “ex vivo” results showing enhanced Ca\(^{2+}\)-induced MPT in rat liver mitochondria of tetrandrine-treated rats (Yan et al., 2006), we can say that our results were confirmed using three different methods (swelling, TPP\(^{+}\)-electrode, and Calcium Green 5-N), and MPT induction was confirmed by CsA. We also used a Ca\(^{2+}\)/protein ratio of 80 nmol Ca\(^{2+}\)/mg protein. The results reported by others (Yan et al., 2006) only used mitochondrial swelling and a very different Ca\(^{2+}\)/protein ratio (75 nmol Ca\(^{2+}\)/5 µg protein), not using CsA to clearly confirm mitochondrial swelling dependent on MPT induction. Thus, the high Ca\(^{2+}\)/protein ratio they used in the swelling assays (Yan et al., 2006) can putatively be due to mitochondrial membrane damage by Ca\(^{2+}\) toxicity rather than MPT induction.

Our results, using isolated rat liver mitochondria, showing tetrandrine protection from ROS and MPT induction, suggest that the compound may protect cells from oxidative stress-induced apoptosis, in quite agreement with other authors (Seow et al., 1988; Matsumo et al., 1990; Castranova, 1994; Ren et al., 1995; Hui et al., 1996; Shen et al., 1999a,b, 2001; Koh et al., 2003; Liu et al., 2004; Sekiya et al., 2005). However, others showed that tetrandrine-induced apoptosis associated with oxidative stress and MPT induction in both cancer cell lines (Jin et al., 2002; Oh and Lee, 2003; Jiang et al., 2004) and rat primary hepatocytes (Yan et al., 2006). This discrepancy suggests that tetrandrine-induced apoptosis is due to factors other than a direct immediate interaction of tetrandrine with isolated mitochondria. The most plausible hypothesis is concerned with the possibility that, at the cellular level, apoptotic factors can be induced by some tetrandrine metabolite (Wu et al., 2004).

Tetrandrine concentrations higher than 100 nmol/mg mitochondrial protein interfere with mitochondrial bioenergetics inducing some stimulation of succinate-supported respiration in state 4 and some inhibition in state 3. This is putatively a consequence of a small permeabilization of mitochondrial inner membrane to H\(^{+}\) and K\(^{+}\) (Fig. 6). This permeabilization, associated with slight inhibition of electron flow in the respiratory chain, explains the extensive decrease of RCR simultaneous with a slight decrease of ADP/O. In fact, oxidative phosphorylation efficiency is insensitive to small specific permeabilization to protons, responsible for a considerable decrease of RCR (Fig. 6B), a fact that can be demonstrated by adding very low concentrations of a protonophore, such as dinitrophenol (results not shown). These results obtained with high concentrations of tetrandrine show that, considering its broad toxicological effects, this concentration range must be avoided to attain its pharmacological actions.

In conclusion, it should be emphasized that our studies analyze a direct immediate action of tetrandrine on mitochondrial mechanisms. Low concentrations of tetrandrine (100 nmol/mg protein) afford protection against liver mitochondrial injury promoted by oxidative stress events, such as ROS production, lipid peroxidation, and Ca\(^{2+}\)-induced MPT. These results may be relevant to its multiple pharmacological actions. Toxicological effects of tetrandrine on the direct mitochondrial metabolism only occur at high concentrations (>100 nmol/mg protein), acting to permeabilize membranes to H\(^{+}\) and K\(^{+}\), and to inhibit mitochondrial electron transfer in the respiratory chain.

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M.A.S. Fernandes et al. / Mitochondrion 6 (2006) 176–185
185


