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Mangiferin, a natural occurring glucosyl xanthone, increases susceptibility of rat liver mitochondria to calcium-induced permeability transition

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Abstract

Mitochondrial permeability transition (MPT) is a Ca^{2+}-dependent, cyclosporine A-sensitive, non-selective inner membrane permeabilization induced by a wide range of agents or conditions, which has often been associated with necrotic or apoptotic cell death. When mitochondria isolated from livers of rats treated with the natural occurring glucosyl xanthone mangiferin (40 mg/kg body weight) were exposed in vitro to Ca^{2+}, they underwent CsA, NEM, and ADP-sensitive high amplitude swelling and associated membrane potential dissipation, release of pre-accumulated Ca^{2+}, oxidation of thiol groups, and depletion of GSH, without changes in the NAD(P)H redox state. The same treatment reduced the phosphorylation rate of mitochondria and the resting respiration by around 4 and 11%, respectively, as well as generation of reactive oxygen species (ROS) by organelle. The in vitro exposure of untreated mitochondria to mangiferin plus Ca^{2+} also resulted in oxidation of thiol groups, in the same way that the compound inhibited the Ca^{2+}-induced peroxidation of mitochondrial membrane lipids. The spectrum of mangiferin during its oxidation by the H_2O_2/HRP system showed a characteristic absorption peak at 380 nm, which decreased immediately after reaction was started; two isosbestic points at around 336 and 412 nm, with a blue shift in the position of the maxima absorption of mangiferin were observed, suggesting their conversion into one oxidation product. Glutathione abolished this decrease of absorbance, suggesting that the oxidation product of mangiferin forms adducts with GSH. We propose that Ca^{2+} increases levels of mitochondria-generated ROS, which reacts with mangiferin producing quinoid derivatives, which in turn react with the most accessible mitochondrial thiol groups, thus triggering MPT. It seems probable that the free radical scavenging activity of mangiferin shifts its anti-oxidant protection to the thiol arylation. An interesting proposition is that accumulation of mangiferin quinoid products would take place in cells exposed to an overproduction of ROS, such as cancer cells, where the occurrence of MPT-mediated apoptosis may be a cellular defence mechanism against excessive ROS formation.

Keywords: Mangiferin; Catechol; Thiol groups; Anti-oxidant; Lipid peroxidation; Mitochondria; Cytotoxicity

Mangiferin, 1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside, is one of the xanthone derivatives and C-glucosylxanthones [1]. This compound is widely distributed in higher plants such as the Anacardiaceae and Gentianaceae families, especially in the leaves and the bark. In addition to its well-documented anti-oxidant properties [2,3], mangiferin has a wide range of pharmacological activities, including anti-diabetic [4], anti-HIV [5], anti-cancer [6], immunomodulatory [7], and anti-inflammatory [8].

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The stem bark of *Mangifera indica* L (Mango) is a predominant source of mangiferin; some time ago, it was widely used in Cuba for treatment of menorrhagia, scabies, diarrhea, syphilis, diabetes, anemia, and cutaneous infections [9]. The extract of this plant, Vimang, is now produced in industrial scale and used in Cuba as a nutritional supplement, in cosmetic products and phytomedicine.

Mangiferin bears a catechol moiety, which is a pharmacophore with well-established anti-oxidant property, also present in catecholamines and numerous flavonoids like quercetin [10]. They are scavengers of highly reactive species such as peroxynitrite and the hydroxyl radical [10–12]. This activity gives rise to the oxidized products semiquinone radicals and quinones, which present potential toxicity due to their oxidant capacity [13–15]. In this regard, it is well-known that weak electrophiles like quinones can deplete cellular thiols both via Michael addition reaction to form dithioethers and via peroxide-mediated oxidation to disulfides, the latter reflecting their susceptibility to redox cycle to liberate reactive oxygen species [16]. Both mechanisms enhance the probability of mitochondrial permeability transition (MPT),[1] which is a cyclosporine A (CsA)-sensitive process mediated by the opening of membrane channels, named permeability transition pores, causing mitochondrial swelling/inner transmembrane potential dissipation. It may be triggered by the concerted action between Ca^{2+} and reactive oxygen species (ROS) leading to oxidation of membrane protein thiols [17] or directly, by hydrophilic dithiol reagents [18–22].

Recent reports have shown that the mangiferin-enriched extract from *Cratoxylum cochinchinense* (YCT) is selectively cytotoxic to Jurkat T cells by a mechanism involving remarkably oxidative stress, MPT, release of cytochrome *c* from mitochondria, and ATP depletion [23]. It was also shown that mangiferin potentiates apoptosis [24] and we found (unpublished results) that the mangiferin-enriched extract Vimang, at 13 μg/ml, causes a 50% reduction in viability of LNCaP human prostate cancer cells.

Taking into account a potential involvement of energy-linked mitochondria functions in this cytotoxicity, the present study addresses the effects of mangiferin mainly on in vivo MPT onset. The results suggest that mangiferin oxidation products trigger MPT via direct interaction with mitochondrial protein thiol groups without affecting the NAD(P)H pool. Mechanistic aspects and potential applications are discussed.

[1] *Abbreviations used:* MPT, mitochondrial permeability transition; RCR, respiratory control ratio; CsA, cyclosporine A; DMSO, dimethylsulfoxide; NEM, *N*-ethylmaleimide; SOD, superoxide dismutase; GSH, glutathione, reduced form; MDA, malonyldialdehyde; RR, ruthenium red; $\Delta\Psi$, membrane potential; ROS, reactive oxygen species; HRP, horseradish peroxidase; RLM, rat liver mitochondria; Hepes, 2-hydroxyethyl-1-piperazineethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Materials and methods

Reagents and solutions

Mangiferin, bovine serum albumin (BSA), ADP, cyclosporine A, EGTA, ascorbic acid, glutathione (GSH), malate, pyruvate, α-ketoglutarate, safranine *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (Hepes), 5,5′-dithiobis(2-nitrobenzoic)acid, *o*-phthalaldehyde, horseradish peroxidase (HRP), superoxide dismutase (SOD), thiobarbituric acid, arsenazo III, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were purchased from Sigma (St. Louis, MO). All other reagents were commercial products of the highest purity grade available. For in vitro experiments, stock solutions of mangiferin in dimethyl sulfoxide were used with 1/1000 dilutions in assay medium to minimize solvent interference.

Animals and mangiferin treatment

Male Wistar rats (4-month-old; approx. 250 g) received food and water *ad libitum*. They were kept in plastic cages with wire tops in a light-controlled room (12 h light–dark cycle) at 22 ± 2 °C. They received a single dose of mangiferin (40 mg/kg body weight) by gavage or water for the controls. Two and half hours later, the animals were killed by cervical dislocation and livers were rapidly extracted. The experiments were in accordance with the Guidelines on the Handling and Training of Laboratory Animals published by the Universities Federation for Animal Welfare (1992).

Isolation of rat liver mitochondria and standard incubation procedure

Mitochondria were isolated by conventional differential centrifugation from the liver of adult animals fasted overnight [25]. The livers were homogenized in 250 mM sucrose, 1 mM EGTA, and 10 mM of Hepes buffer (pH 7.2). The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was resuspended in 250 mM sucrose to a final protein concentration of 80–100 mg/ml, measured by the method of Biuret with BSA as protein standard [26].

The experiments were carried out in standard medium containing 125 mM sucrose, 65 mM KCl, 2 mM inorganic phosphate, 1 mM magnesium chloride, 10 mM of Hepes buffer (pH 7.2), and a cocktail of NAD-linked substrates at 5 mM (malate, pyruvate, and α-ketoglutarate). Other additions are indicated in the figure legends.

Mitochondria respiration rates and phosphorylation efficiency

Oxygen consumption in mitochondrial suspensions was polarographically determined with a Clark-type electrode (Yellow Springs Instruments Co.) in a 1.3 ml glass chamber equipped with a magnetic stirrer at 28 °C. Liver mitochondria (1 mg protein/ml) were added to standard respiration medium. Respiration rates are given in ng atom oxygen/mg/min. Phosphorylating (state III) respiration was initiated by addition of 200 nmol ADP/mg protein. Phosphorylation efficiency (ADP/O ratio) was calculated from the added amount of ADP and total amount of oxygen consumed during state III. The experimental approach was calibrated using the oxygen content of air saturated medium, 425 ng atoms/ml at 28 °C [27].

Measurement of mitochondrial transmembrane electrical potential ($\Delta\Psi$)

The mitochondrial membrane potential was estimated as fluorescence changes of safranin [28], recorded on a model F-4010 Hitachi spectrofluorimeter operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with a slit width of 5 nm. Mitochondria (0.5 mg/ml) were incubated in 2 ml of standard medium supplemented with 5 μM safranine. After 30 s, 5 mM of NAD-linked substrate cocktail was added. When $\Delta\Psi$ was established, 20 μM Ca^{2+} was added to the medium and the times of $\Delta\Psi$ collapses were compared. Relative changes in membrane potential were expressed in arbitrary fluorescence units and were not converted to potential values.

Mitochondrial swelling

Mitochondrial swelling was estimated from the decrease in absorbance of the mitochondrial suspension measured at 540 nm in a Hitachi U-3000 spectrophotometer equipped with magnetic stirring and temperature control (28 °C), essentially as described by Petronilli et al. [29]. Mitochondria were incubated in the standard incubation medium at 0.5 mg of mitochondrial protein/ml.

Determination of protein thiol groups content

Mitochondrial membrane thiol groups were determined using 5,5′-dithiobis(2-nitrobenzoic)acid (Ellman's reagent) as described in [30,31]. The mitochondrial suspension was briefly incubated in standard reaction medium, submitted to three subsequent freeze–thawing procedures to release matrix proteins, and then centrifuged during 2 min at 6708*g* in a 5415 C Eppendorf Centrifuge. The pellet was treated with 200 μl of 6.5% trichloroacetic acid and centrifuged at 6708*g* during 2 min to precipitate the protein. This procedure was repeated two times. The final pellet was suspended in 1 ml of a medium containing 0.5% sodium dodecyl sulfate, 0.5 mM EDTA, 0.5 M Tris, pH 8.3, and 100 μM 5,5′-dithiobis(2-nitrobenzoic)acid. Absorption was measured at 412 nm, using glutathione for calibration. Under control conditions, total mitochondrial membrane protein thiol content was approximately 45 nmol/mg protein.

Determination of NAD(P) redox state

The oxidation or reduction of pyridine nucleotides in the mitochondrial suspension was followed in a Hitachi F-4010 spectrofluorimeter operating at 366 nm excitation and 450 nm emission [32]. All incubations were conducted in the presence of cyclosporine A to avoid fluorescence changes secondary to mitochondrial swelling, release of matrix NAD(P)H, or inhibition of NAD(P)H transhydrogenase activity due to increased membrane proton leakage.

Determination of mitochondrial GSH

The mitochondrial suspension (1 ml) was treated with 0.5 ml of 13% trichloroacetic acid and centrifuged at 900*g* for 3 min. Aliquots (100 μl) of the supernatant were mixed with 2 ml of 100 mM NaH_2PO_4 buffer, pH 8.0, containing 5 mM EGTA. One hundred microliters of *o*-phthalaldehyde (1 mg/ml) was added and fluorescence was measured 15 min later using the 350/420 nm excitation/emission wavelength pair with a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) [33].

Reactive oxygen species production

Production of reactive oxygen species (ROS) by liver mitochondria was monitored using the Amplex Red assay for H_2O_2 (Molecular Probes, Eugene, OR) [34]. Mitochondria (0.5 mg/ml) were incubated in standard incubation medium supplemented with 50 μM Amplex Red and 0.025 μM U/ml horseradish peroxidase at 28 °C, with continuous stirring. Fluorescence of oxidized probe was measured in a Hitachi F-4010 spectrofluorimeter at 563 nm for excitation and 587 nm for emission.

Lipid peroxidation assay

Lipid peroxidation was estimated from malonyldialdehyde (MDA) generation [35]. The mitochondrial suspension (1 ml) was incubated with 1 ml of 1% thiobarbituric acid (prepared in 50 mM NaOH), 0.1 ml of 10 M NaOH, and 0.5 ml of 20% H_3PO_4, for 20 min at 85 °C. The MDA–thiobarbituric acid complex was extracted with 2 ml *n*-butanol and absorbance of the extract was measured at 535 nm. MDA concentration was calculated from $\varepsilon = 1.56 \times 10^5\,M^{-1}$.

Evaluation of Ca^{2+} movements

Variations in free Ca^{2+} concentration were followed by measuring the changes in the absorbance spectrum of the metallochromic indicator arsenazo III [36], using an SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm at 28 °C.

One-electron oxidation of mangiferin (activation)

The oxidation of mangiferin by horseradish peroxidase (HRP) was followed spectrophotometrically by recording scan from 300 to 600 nm every 2 min. The reaction was initiated by the addition of HRP (1.6 nM) to the incubation mixture containing mangiferin (50 μM) and H_2O_2 (50 μM) without or in the presence of 100 μM GSH or 50 μM ascorbate. All spectrophotometric experiments were performed in phosphate buffer (10 mM, pH 7.2) at 28 °C.

Statistics

Statistical analysis was performed by the unpaired Student's *t* test. All experiments were performed at least in triplicate, using different mitochondrial preparations. Data are given as means ± SD or as a typical example.

Results

The oxygen consumption by isolated rat liver mitochondria exposed to NAD^+-dependent substrates is presented in Fig. 1. Respiration rates were measured in the presence of added ADP (V_3) or in its absence (V_4) (A). The yield of oxidative phosphorylation was determined by ADP/O ratio, i.e., the moles of ADP phosphorylated by atom gram of oxygen consumed (B). The addition of 20 μM Ca^{2+} to untreated mitochondria (control) did not affect V_3 respiration, but increased V_4 ($p < 0.05$) with a consequent reduction of RCR ($p < 0.05$) and the ADP/O ratio ($p < 0.05$). The treatment of rats with mangiferin reduced the mitochondrial phosphorylation rate (V_3 or state III respiration) by around 4% ($p < 0.05$). It also reduced the resting respiration (V_4 or state IV respiration) by 11% ($p < 0.05$). The overall effect of mangiferin on mitochondrial respiration did not affect the phosphorylation efficiency (ADP/O ratio). Addition of Ca^{2+} to mitochondria isolated from mangiferin-treated rats inhibited V_3 respiration by around 35% ($p < 0.01$) and increased V_4 by around 23% ($p < 0.05$), reducing RCR of mitochondria ($p < 0.01$) and also its phosphorylating capacity ($p < 0.01$). The effects of mangiferin treatment on the isolated mitochondria exposed to a FAD-dependent substrate (5 mM potassium succinate plus 1 μg/ml rotenone, results not shown) were very similar to the effects on mitochondria exposed to the NAD^+-dependent substrates described above. Together, these results suggest that mangiferin synergizes the deleterious Ca^{2+} effects on mitochondria.

It is well-documented that in resting respiration, there is a maximum ROS generation by mitochondria [37], so that mangiferin could be scavenging superoxide radicals formed by oxygen reception of electrons leaked from highly reduced mitochondrial respiration complexes (I, II, and III). Mangiferin interference in electron transport

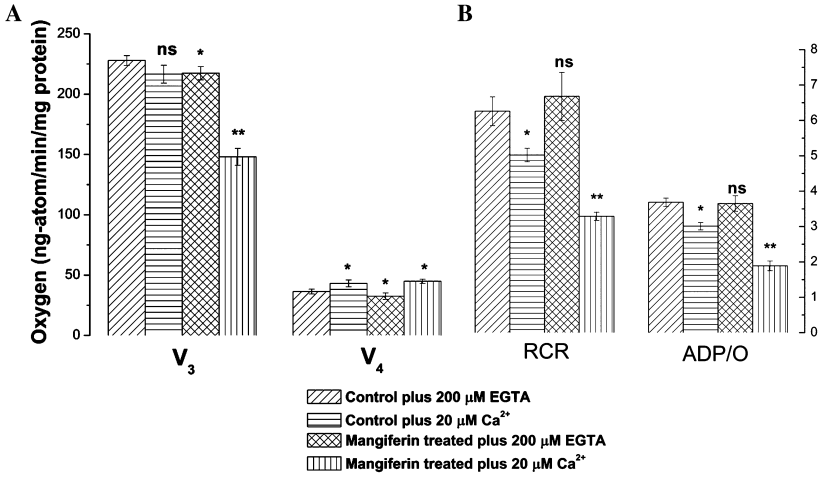

Fig. 1. (A) Effect of in vivo mangiferin treatment on phosphorylating (V_3) and resting (V_4) respiration rates of rat liver mitochondria. Respiration rates were expressed in nanograms-atoms of oxygen consumed per minute and per milligram of mitochondrial protein. Data were means ± SD ($n = 3$). Mitochondria were incubated at 28 °C in incubation medium containing 125 mM sucrose, 65 mM KCl, 2 mM inorganic phosphate, 1 mM magnesium chloride, 0.2 mM EGTA, and 10 mM of Hepes buffer at pH 7.2 with a cocktail of NAD-linked substrates at 5 mM (malate, pyruvate, and α-ketoglutarate). For V_3 determination, 200 μM ADP was added. V_4 was determined after ADP depletion. (B) Respiration control ratio (RCR) was determined dividing V_3/V_4; phosphorylation efficiency (ADP/O ratio) was calculated from the added amount of ADP and total amount of oxygen consumed during ADP pulse. The bars show means ± SD ($n = 3$ independent mitochondrial preparations). One tailed *t* test was used for statistical analyses; $^*p < 0.05$, $^{**}p < 0.01$, and ns, no significant respect to control (untreated).

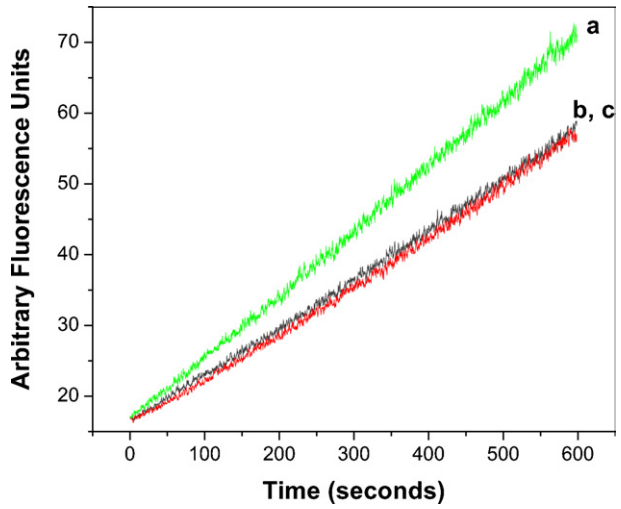

Fig. 2. H_2O_2 production monitored by Amplex Red fluorescence. Mitochondria (0.5 mg/ml) were incubated as described under Materials and methods. (a) Mitochondria isolated from untreated animals (controls) plus 20 μM Ca^{2+}, (b) mitochondria isolated from mangiferin-treated rats plus 20 μM Ca^{2+}, (c) control mitochondria plus 0.2 mM EGTA. The assays were performed three times with different mitochondria preparations with comparable results.

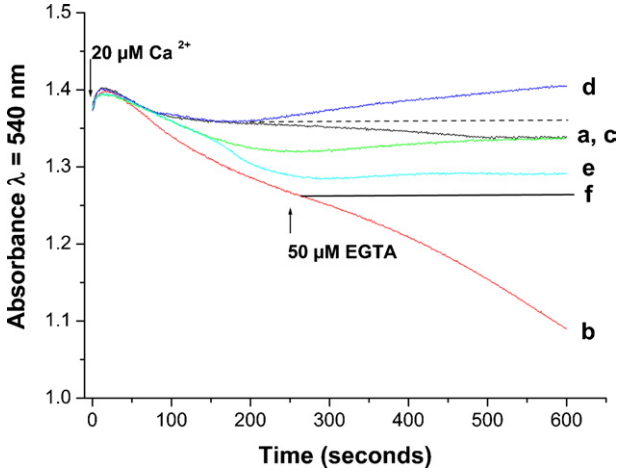

Fig. 3. Calcium-dependent mitochondrial swelling induced by in vivo treatment with mangiferin. Rat liver mitochondria (RLM, 0.5 mg/ml) were incubated in standard medium supplemented with 20 μM Ca^{2+} under the conditions indicated in Materials and methods. A downward deflection indicates mitochondrial swelling. Reaction medium contains: (a) control mitochondria (from untreated rats), (b) mitochondria isolated from mangiferin-treated rats, (c) mitochondria from mangiferin-treated rats plus 1 μM cyclosporine A (CsA), (d) mitochondria from mangiferin-treated rats plus 20 μM *N*-ethylmaleimide (NEM), (e) mitochondria from mangiferin-treated rats plus 500 μM ADP and 1 μg/ml oligomycin, and (f) mitochondria from mangiferin-treated rats plus 50 μM EGTA (added 4 min after Ca^{2+}). Dashed line represents mitochondria from mangiferin-treated rats with 1 μM ruthenium red (RR) or 500 μM EGTA (initially added). Ca^{2+} (20 μM) and 50 μM EGTA were added where indicated by the arrows. Three additional experiments with different mitochondrial preparation exhibited the same trend.

from complexes I/II to oxygen could also reduce free radical production in state III respiration, which could yield a net less ROS mitochondria production. To evaluate these possibilities, we measured the H_2O_2 released in medium by using the Amplex Red assay, in the absence or presence of 20 μM Ca^{2+}, a condition inducing mitochondrial swelling. Fig. 2 shows that mitochondria from mangiferin-treated rats (b) release less hydrogen peroxide than mitochondria from untreated (controls) rats (a). Furthermore, EGTA reduced the rate of H_2O_2 production of controls exposed to Ca^{2+}.

Suspension of liver mitochondria from rats treated with mangiferin (40 mg/kg oral mangiferin dose), exposed in vitro to 20 μM Ca^{2+}, presented a change in apparent absorbance in relation to controls, consistent with large amplitude swelling (Fig. 3), which was accompanied by membrane depolarization (Fig. 4) and release of pre-accumulated Ca^{2+} (Fig. 5). All these effects were almost completely inhibited by the classical MPT modulators CsA (1 μM), NEM (20 μM) or ADP (500 μM). The Ca^{2+} chelator, EGTA, and the mitochondrial Ca^{2+} uniporter inhibitor, ruthenium red, also prevented the mitochondrial swelling, indicating, as believed, a Ca^{2+} dependence for the process. Only NEM was able to completely recover the initial absorbance values of the mitochondrial suspension (Fig. 3, line d), suggesting the involvement of thiol groups of mitochondrial membrane in these effects. Indeed, Fig. 6 shows that Ca^{2+} stimulated a decrease of protein thiols in mitochondria from the mangiferin-treated rats, and that this decrease was completely inhibited by EGTA and ruthenium red, but not by cyclosporine A. These results suggest a mutual or synergistic effect between the mangiferin treatment of rats and the in vitro exposition of mitochondria to Ca^{2+} on the decrease of mitochondrial membrane protein thiols. Apparently, in vivo exposition of mitochondria to mangiferin makes isolated organelle more sensitive to these Ca^{2+} effects relative to untreated (controls) mitochondria.

The in vitro exposure of untreated mitochondria to growing mangiferin concentration in the absence or presence of 20 μM Ca^{2+} attests the requirement of Ca^{2+} for the decrease of mitochondrial thiols by mangiferin. Thus, Fig. 7A shows that while in the presence of 200 μM EGTA, mangiferin was unable to decrease the concentration of mitochondrial reduced thiols, in the presence of 20 μM Ca^{2+}, without EGTA, this effect was potentialized in a dose-dependent manner, apparently on account of its anti-oxidant activity (see Fig. 2). In this context, Fig. 7B shows that mangiferin inhibited Ca^{2+}-induced lipid peroxidation, also in a dose-dependent manner, suggesting that the anti-oxidant activity of mangiferin may shift the radical damage from the mitochondrial lipid peroxidation to the thiol diminution.

Fig. 8 shows the effect of Ca^{2+} on the mitochondrial GSH levels of liver mitochondria isolated from

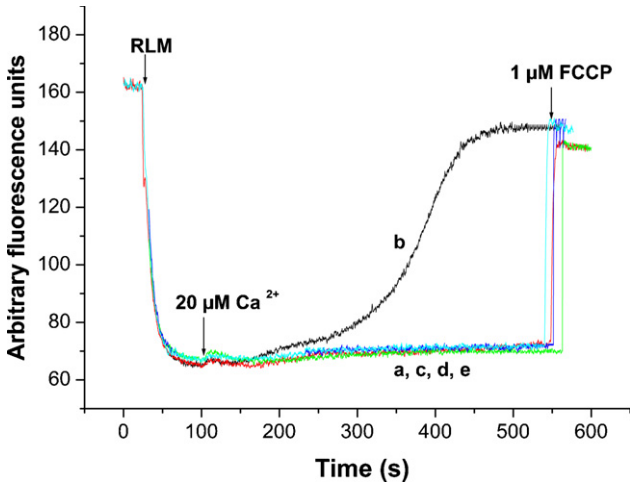

Fig. 4. Mangiferin stimulates Ca^{2+}-dependent loss of mitochondrial potential ($\Delta\Psi$) expressed as Arbitrary Fluorescence Units. Experimental conditions are described under Materials and methods. Reaction medium contains: (a) mitochondria from untreated rats (control) (b) mitochondria from mangiferin-treated rats, (c) mitochondria from mangiferin-treated rats plus 1 μM CsA, (d) mitochondria from mangiferin-treated rats plus 20 μM NEM, and (e) mitochondria from mangiferin-treated rats plus 500 μM ADP and 1 μg/ml oligomycin. RLM (0.5 mg/ml), 20 μM Ca^{2+}, and 1 μM FCCP were added where indicated by the arrows. Results are representative of three experiments.

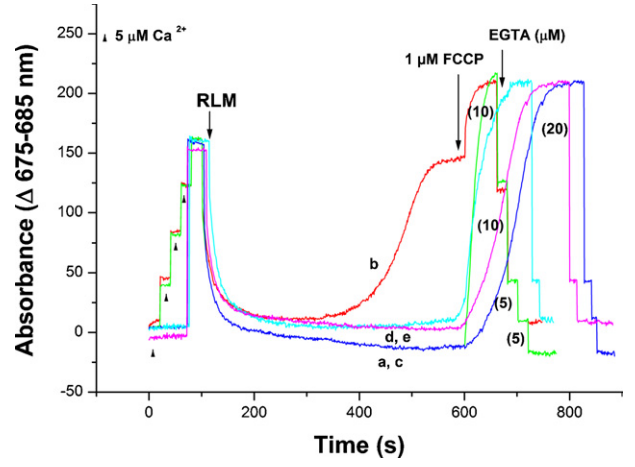

Fig. 5. Effect of in vivo mangiferin treatment on Ca^{2+} retention by rat liver mitochondria. Rat liver mitochondria (0.5 mg/ml) were incubated in standard reaction medium containing 40 μM arsenazo III. Experimental conditions are as described under Materials and methods: (a) mitochondria from untreated rats (Control), (b) mitochondria from mangiferin-treated rats, (c) mitochondria from mangiferin-treated rats plus 1 μM CsA, (d) mitochondria from mangiferin-treated rats plus 20 μM NEM, and (e) mitochondria from mangiferin-treated rats plus 500 μM ADP and 1 μg/ml oligomycin. Rat liver mitochondria (RLM), 20 μM Ca^{2+}, EGTA (concentration between parentheses), and 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were added where indicated by the arrows. The assays were performed three times with different mitochondrial preparation and comparable results were obtained.

mangiferin-treated or untreated (control) rats. In agreement with the results on Figs. 6 and 7, in vitro exposition of mitochondria from the mangiferin-treated rats to

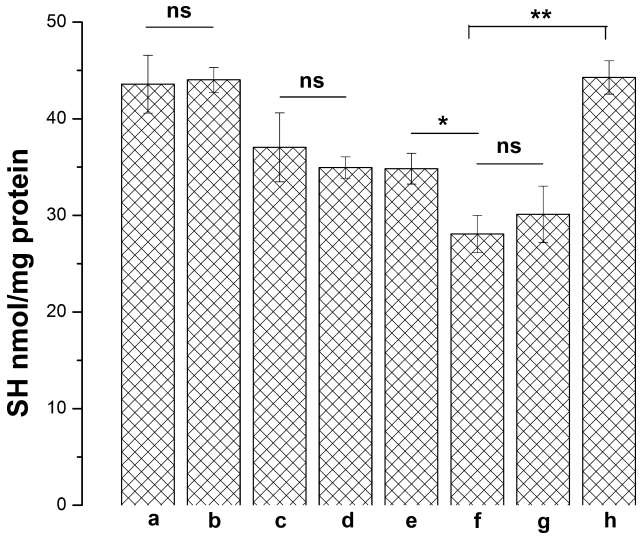

Fig. 6. Calcium stimulates a reduction of mitochondrial membrane protein thiol content in mangiferin-treated rats. Rat liver mitochondria (0.5 mg/ml) were incubated in reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM of Hepes buffer, pH 7.2, 5 mM α-ketoglutarate, 5 mM malate, and 5 mM piruvate: (a) mitochondria from untreated (control) rats plus EGTA, (b) mitochondria from mangiferin-treated rats plus EGTA, (c) control plus 10 μM Ca^{2+}, (d) mitochondria from mangiferin-treated rats plus 10 μM Ca^{2+}, (e) control plus 20 μM Ca^{2+}, (f) mitochondria from mangiferin-treated rats plus 20 μM Ca^{2+}, (g) mitochondria from mangiferin-treated plus 20 μM Ca^{2+} plus 1 μM CsA, and (h) mitochondria from mangiferin-treated rats plus 20 μM Ca^{2+} plus RR. Results were expressed as means ± SD ($n = 3$ independent mitochondrial preparations). One tailed *t* test was used for statistical analyses; $*p < 0.05$ and ns, no significant.

20 μM Ca^{2+}, depleted the GSH pool, an effect completely dependent on the presence of Ca^{2+} and not observable in mitochondria of controls.

Since pyridine nucleotides and thiol groups are in oxidation–reduction equilibrium through pyridine nucleotides transhydrogenase, glutathione reductase, and possibly mitochondrial thioredoxin reductase [38], we evaluated the NAD(P)H redox status of mitochondria isolated from mangiferin-treated and untreated (control) rats, no significant oxidation being observed (data not shown).

Prooxidant effects similar to those of mangiferin on mitochondria have been previously reported in relation to the activation of polyphenols to semiquinone/quinone derivatives by nitric oxide [39], which could display the same role as that of Ca^{2+} in our system. Because the in vivo effects of Ca^{2+} on respiring mitochondria from mangiferin-treated rats could be related with the oxidation/activation of the xanthone, we evaluated the effect of the oxidizing HRP/H_2O_2 system in the mangiferin spectrum in presence of biological anti-oxidants like ascorbate, GSH, and SOD; this system is able to induce one electron oxidation of many substrates [40]. Hence, Fig. 9A shows the spectrum of mangiferin (50 μM)

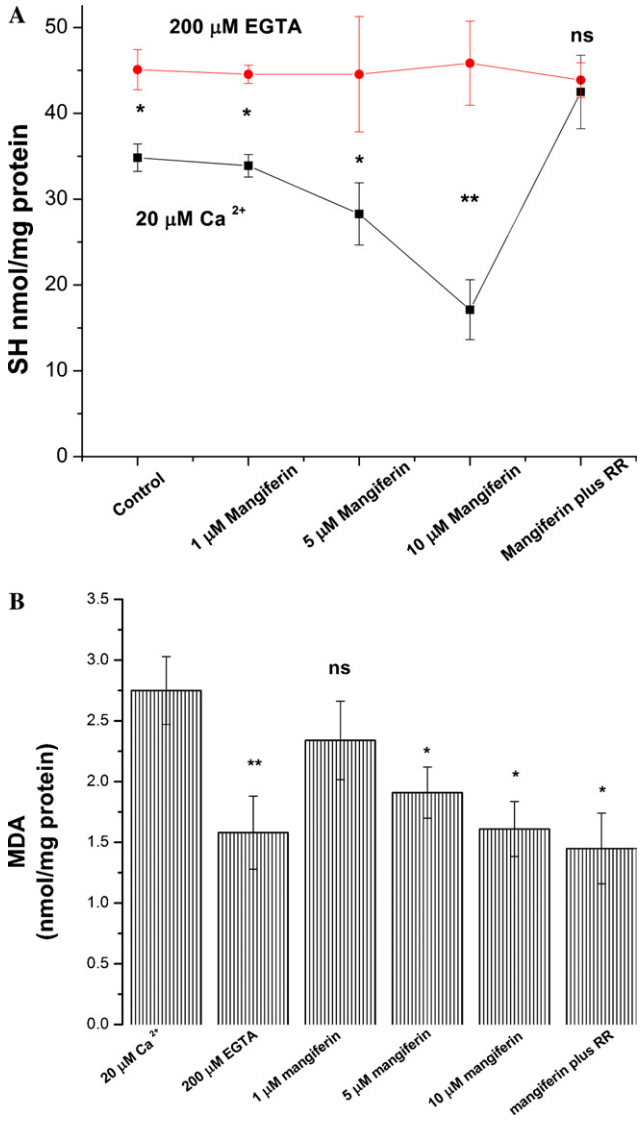

Fig. 7. Calcium dependence of in vitro mangiferin reduction of mitochondrial membrane protein contents and lipid peroxidation. Experimental conditions are as described in Fig. 1. Rat liver mitochondria (0.5 mg/ml) were incubated with mangiferin (1, 5, and 10 μM) or 10 μM mangiferin plus 1 μM RR for 10 min in the presence of 20 μM Ca^{2+} (■) or 200 μM EGTA (●). Membrane protein thiol content and malondialdehyde (MDA) were determined as described under Materials and methods. The symbols and the bars show means ± SD ($n = 3$ independent mitochondrial preparations). Controls contain only DMSO (14.3 μM), which is the solvent concentration in the mangiferin samples. One tailed t test was used for statistical analyses; $^{*}p < 0.05$, $^{**}p < 0.01$, and ns, not significant.

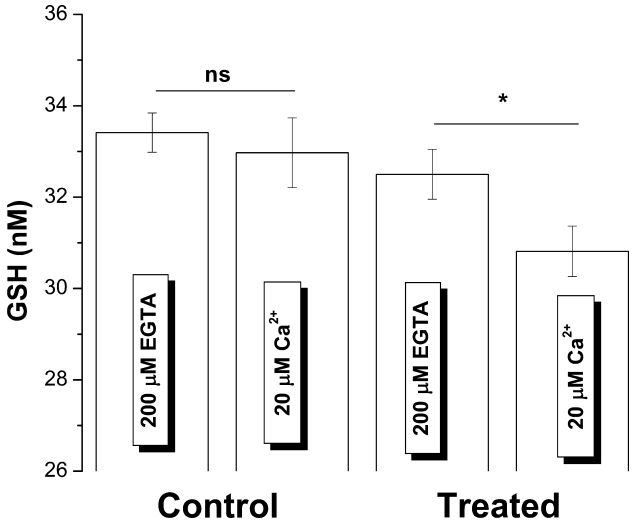

Fig. 8. Influence of Ca^{2+} on mitochondrial glutathione (GSH) of mangiferin-treated (treated) or untreated (control) rats. Experimental conditions are as described in Fig. 1. RLM from mangiferin-treated rats or control rats (1 mg/ml) were incubated 10 min in the presence (20 μM) or in the absence (200 μM EGTA) of Ca^{2+}. GSH was determined according to Materials and methods description, interpolating the absorbance values in a calibration curve with standard GSH. The bars show means ± SD ($n = 3$ independent mitochondrial preparations). One tailed t test was used for statistical analyses; $^{*}p < 0.05$ and ns, not significant.

during its oxidation by HRP. A characteristic absorption peak at 380 nm, corresponding to the B-ring, decreased immediately after reaction was started (Fig. 9A, scan b). Two isosbestic points at around 336 and 412 nm can be observed, with a small, but measurable blue shift in the position of the maxima absorption of mangiferin (375 nm), suggesting its conversion into one oxidation product. By analogy with literature data [14,41], a potential candidate is the *o*-quinone structure (mQ), which is stable upon prolonged incubation in the reaction mixture (60 min). The addition of 50 μM ascorbate or 4 U SOD to the reaction mixture did not prevent the accumulation of this product (Figs. 9B and D), suggesting that the *o*-semiquinone radical formed interacts with other *o*-semiquinone radical rather than with ascorbate or O_2. The addition of 100 μM GSH developed a family of spectra, which was not isosbestic with the mangiferin spectrum, suggesting that another product was formed (Fig. 9C). On the other hand, glutathione inhibited the H_2O_2/HRP-induced decrease of absorbance of mangiferin at 380 nm, suggesting that once mQ is created (or during its formation), it forms adducts with GSH. It is noteworthy observe that mangiferin did not interact with GSH or H_2O_2 in the absence of HRP (results not shown), meaning that oxidation/activation of this compound is required for such interaction.

Discussion

The results presented in this study show that when mitochondria isolated from livers of rats treated with mangiferin are exposed in vitro to Ca^{2+}, they undergo respiration inhibition and uncoupling (Fig. 1), high amplitude swelling (Fig. 3) and associated membrane potential dissipation (Fig. 4), release of pre-accumulated Ca^{2+} (Fig. 5), and oxidation of thiol groups (Figs. 6 and 8),

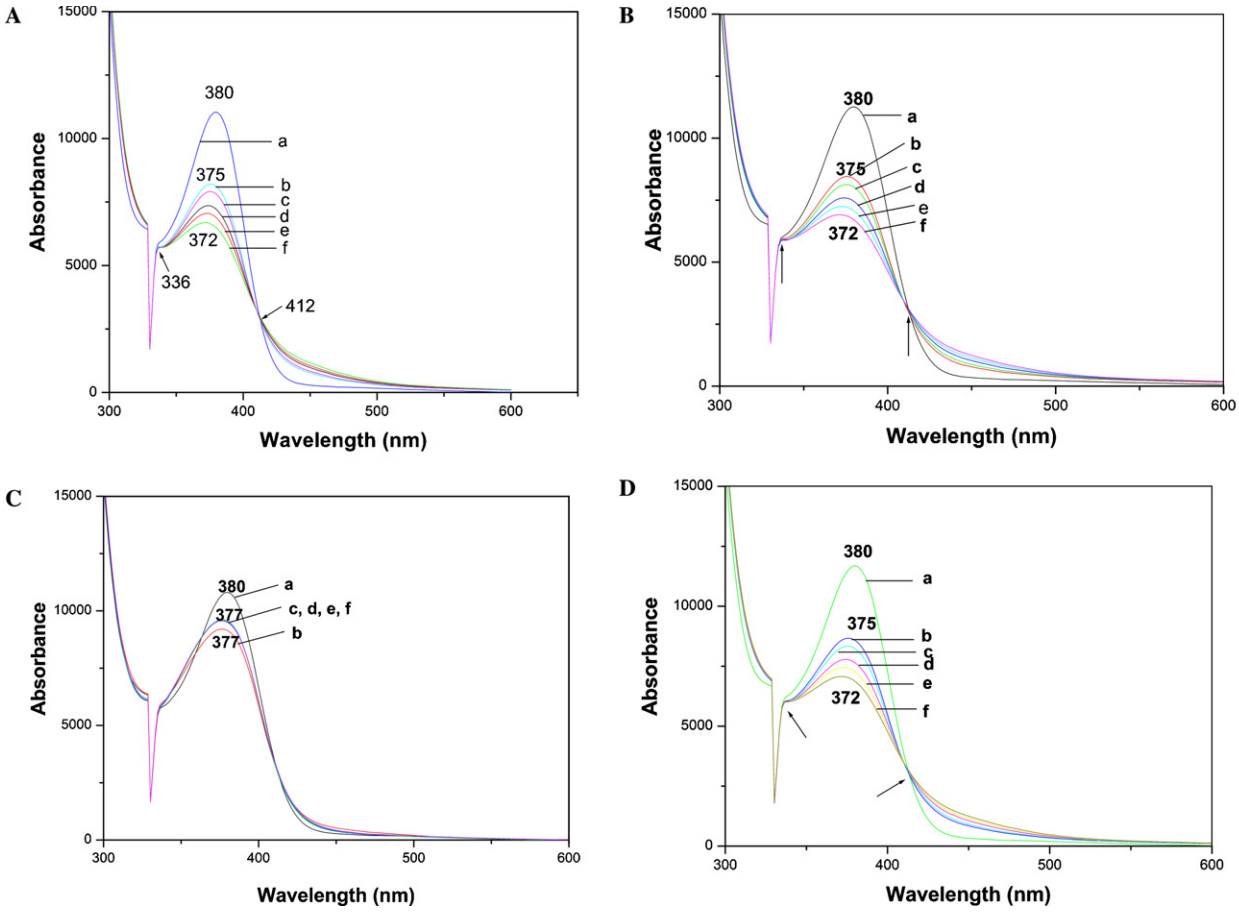

Fig. 9. Time-course changes of visible absorption of 50 μM mangiferin during the horseradish peroxidase (HRP)-catalyzed oxidation of mangiferin (A) and the effect of 50 μM ascorbate (B), 100 μM GSH (C) or 4 U SOD (D) added before HRP. The reaction mixture contained 1.6 nM HRP, 50 μM mangiferin, and 50 μM H_2O_2 in 10 mM phosphate buffer, pH 7.2. The reaction was started by the addition of HRP. The scans were started immediately (a), 1 min (b), 5 min (c), 10 min (d), and 20 min (e) after the addition of HRP. Scan speed was 600 nm/min. The incubation of mangiferin with H_2O_2, GSH, ascorbate or superoxide dismutase (SOD) (20 min), without HRP addition, does not modify the mangiferin spectrum. Shown is a typical experiment; three additional experiments yielded comparable results.

without changes in NAD(P)H redox state. All these events are inhibited by the classical MPT modulators CsA, a ligand of mitochondrial cyclophilin, NEM, a hydrophobic monofunctional thiol reagent, and ADP, which alter conformation of the ADP/ATP translocator [42,43]. Swelling induction, in particular, is prevented by EGTA and ruthenium red (Fig. 3), indicating, as believed, a strict dependence of the phenomenon by Ca^{2+}. While this effect is paradoxical for a compound having potent anti-oxidant, anti-inflammatory, and cytoprotective properties [2,3,8], it could explain its proapoptotic and antiproliferative activities observed in different cell lines [6,23,24]. MPT is considered as one of the key processes responsible for cell killing by oxidants, mainly via apoptosis induction. The process is accompanied by membrane depolarization, loss of ion and volume regulation, and inhibition of oxidative phosphorylation [44]. In addition to oxidants, many other xenobiotics are known to induce MPT in vitro, including transition metals and soft electrophiles, which are able to form thioether adducts via Michael addition reactions.

Insights into possible mechanisms of MPT modulation may be inferred from the chemical reactivity of recognized inducers of MPT in vitro, such as hydroperoxides and sulfhydryl reagents [32,45]. The current hypothesis is that hydroperoxides, through the concerted action of glutathione peroxidase and membrane transhydrogenase, causes oxidation of critical protein thiols to disulfides, resulting in MPT onset.

Mangiferin is an effective anti-oxidant, mainly on account of its catechol moiety [2,3], which is present also in numerous flavonoids and other polyphenols. During protection afforded against free radical production, the catechols are oxidized, generating products like semiquinone radicals and quinones. These compounds have been described to be potentially toxic, especially in virtue of their ability to arylate protein thiol groups [15,46]. Indeed, a reference quinone, i.e., 4-methylortho-benzoquinone, is able to react with protein thiols as efficiently as the synthetic sulfhydryl-alkylating agent NEM. This observation suggests that the oxidation products of catechol-containing anti-oxidants like mangiferin shift the

damage provoked by oxidative stress to sulfhydryl arylation [47].

MPT induced by thiol cross-linkers is independent of ROS, occurring in virtue of thiol cross-linkage promoted directly by the inducer [32,45]. In this regard, our results suggest that the basal generation of ROS by mitochondria does not produce sufficient mangiferin oxidized product to induce mitochondrial damage through thiol interactions, so that it needs an activator like Ca^{2+}. Recent studies have shown that Ca^{2+} stimulates electron leakage (ROS production) at the level of the reduced and semiquinone forms of coenzyme Q [45,48], due to Ca^{2+} binding to cardiolipins. A consequent disorganization of the lipid phase [49] would alter interactions between electron-transferring molecules, thus favoring electron donation to oxygen at intermediate steps of the respiratory chain [50]. Therefore, our proposal is that exposure of mitochondria from mangiferin-treated rats to Ca^{2+} stimulates the generation of ROS, whose reaction with mangiferin (Figs. 2 and 7B) produces quinoid derivatives which, in turn, react with the most accessible mitochondrial thiol group (Figs 6, 7A, and 8). Indeed, another set of data from this study provides spectral evidence for an enzymatic activation of mangiferin and their possible implication in cellular (mitochondrial) detoxification and toxicity (Fig. 9). Thus, formation of the free radical intermediate *o*-semiquinone in mangiferin catechol moiety during HRP-catalyzed oxidation of mangiferin occurs by a classical peroxidase mechanism [40] and is a prerequisite for the radical stabilization; its mesomeric structure is also an aroxyl radical and involves all the three rings of mangiferin. In this context, the superoxide radical formed by the Ca^{2+} effect on mitochondrial membrane could also react with mangiferin in the same manner as HRP/H_2O_2, activating the compound through its free radical scavenging ability, and shifting anti-oxidant protection to thiol arylation.

Regarding MPT induction by polyphenols, in general, it has been recently demonstrated that curcumin was able to oxidize mitochondrial membrane thiols triggering MPT in presence of low Ca^{2+} concentrations, an effect elicited while the compound acted as a potent anti-oxidant [51]; the proposed mechanism also includes the interaction of curcumin oxidized product with mitochondrial thiol groups. On the other hand, genistein, another poliphenol, was found to induce MPT via ROS generation due to its interaction with respiratory chain at the mitochondrial complex III level, directly reacting with transition metals and O_2. In this regard, MPT induction by genistein was completely prevented when complex I and III (upstream of the inhibitory site of antimycin A) of the respiratory chain were in a reduced state [52].

The ability of some polyphenols to reduce iron ions has often been associated with their prooxidant activities [53]. This however, does not seem to be the case for the mechanisms underlying the effects of mangiferin on mitochondria: we have recently demonstrated that mangiferin and mangiferin-contained extract (Vimang) inhibit Fe(II)-citrate-mediated membrane lipid peroxidation in isolated mitochondria by mechanisms involving stimulation of Fe(II) autoxidation and inhibition of Fe(III) reduction by ascorbate [54,55]. In addition, it seems unlikely that mangiferin or its oxidation products interact directly with complex III or other components of mitochondrial electron transfer chain. In this case, the above mentioned effects of mangiferin on mitochondria would be manifested even in the absence of external Ca^{2+} addition.

Mitochondria are the most important intracellular source of ROS, either under physiological or pathological conditions. Our results suggest that mangiferin accumulation in normal mitochondria does not affect the organelle by itself. Indeed, the slight inhibition of mitochondrial respiration elicited by mangiferin is compensated by the reduction of resting respiration (Fig. 1). However, under conditions in which mitochondrial generation of ROS is increased, such as in the presence of Ca^{2+} or when the mitochondrial anti-oxidant defense is compromised, ROS accumulation may lead to an important accumulation of the mangiferin oxidized products, which directly react with mitochondrial membrane thiol groups, resulting in mitochondrial dysfunction and ultimately cell death. Free radical scavengers afford protection against oxidative stress since they can react with damaging reactive species. Despite this protection, the toxicity of reactive oxidation products generated during the actual anti-oxidant activity might be substantial, e.g., weakening of the endogenous anti-oxidant defence and impairment of vital enzymes.

These aspects should be considered during the utilization of mangiferin or the mangiferin-containing extracts. In this regard, an interesting proposition is that accumulation of mangiferin quinoid products would take place in such cells exposed to an overproduction of ROS, such as cancer cells, where the occurrence of apoptosis induced by MPT may be a cellular defence mechanism against excessive ROS formation. Therefore, mangiferin could stimulate such mechanism, acting as a prodrug, whose toxicity is limited only to the ROS-damaged cells.

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