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Fe(III) Shifts the Mitochondria Permeability Transition-Eliciting Capacity of Mangiferin to Protection of Organelle

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ABSTRACT

Mangiferin acts as a strong antioxidant on mitochondria. However, when in the presence of Ca^{2+}, mangiferin elicits mitochondrial permeability transition (MPT), as evidenced by cyclosporin A-sensitive mitochondrial swelling. We now provide evidence, by means of electrochemical and UV-visible spectroscopical analysis, that Fe(III) coordinates with mangiferin. The resulting mangiferin-Fe(III) complex does not elicit MPT and prevents MPT by scavenging reactive oxygen species. Indeed, the complex protects mitochondrial membrane protein thiols and glutathione from oxidation. Fe(III) also significantly increases the ability of mangiferin to scavenge the 2,2-diphenyl-1-picrylhydrazyl radical, as well as to display antioxidant activity toward antimycin A-induced H_2O_2 production and *t*-butyl hydroperoxide-promoted membrane lipid peroxidation in mitochondria. We postulate that coordination with Fe(III) constitutes a potential protective mechanism toward the prooxidant action of mangiferin and other catechol-containing antioxidants regarding MPT induction. Potential therapeutic relevance of this finding for conditions of pathological iron overload is discussed.

In association with their antioxidant activities, several polyphenols may perform prooxidant actions in different biological systems. In particular, catechol-containing polyphenols are capable of forming electrophilic quinone/quinone methide intermediates during their interaction with reactive oxygen species (ROS). These intermediates may then react with key target molecules such as DNA, proteins, and glutathione (Galati et al., 2001; Awad et al., 2002; Walle et al., 2003). The polyphenol mangiferin (1,3,6,7-tetrahydroxyxanthone-C2-*β*-D-glucoside) displays important pharmacological and antioxidant activities (Sato et al., 1992; Ghosal et al., 1996). This compound contains a catechol moiety capable of forming stable complexes with iron (Ghosal et al., 1996).

We previously demonstrated that mangiferin protects isolated mitochondria from membrane lipid peroxidation (Andreu et al., 2005b) and inhibits oxidative damage to 2-deoxyribose (Pardo-Andreu et al., 2006a), mainly through a mangiferin-iron complex. Indeed, ferric ions improve the antioxidant capacity of several catechols (Moridani and O'Brien, 2001; Moridani et al., 2003; Kostyuk et al., 2004; Mahal et al., 2005). In contrast, we also demonstrated that in the presence of Ca^{2+}, mangiferin elicits mitochondrial permeability transition (MPT) through the arylation of thiol groups of mitochondrial proteins and proposed that mangiferin oxidation products, including quinone-types metabolites, rather than mangiferin itself, elicit this effect (Andreu et al., 2005a).

MPT is a process mediated by the opening of membrane pores in mitochondria, referred to as permeability transition pores, and demonstrated by cyclosporin A (CsA)-sensitive swelling of isolated organelles. It is induced in response to Ca^{2+} plus oxidative stress conditions, a combination that triggers a conformational change in inner mitochondrial membrane proteins; oxidation of both thiol groups of these proteins and glutathione is probably involved (Zoratti and Szabò, 1995; Kowaltowski et al., 2001; He and Lemasters, 2002; Halestrap, 2005).

Taking into account the improving effect of iron on the antioxidant activity of some polyphenols (Moridani and O'Brien, 2001; Moridani et al., 2003; Kostyuk et al., 2004;

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ABBREVIATIONS: ROS, reactive oxygen species; MPT, mitochondrial permeability transition; CsA, cyclosporin A; MDA, malondialdehyde; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; GSH, reduced glutathione.

Mahal et al., 2005), as well as the well established involvement of ROS in MPT (Kowaltowski et al., 2001), we examined here whether iron can influence the pro-oxidant action of mangiferin relating to MPT. We demonstrate that coordination with Fe(III) prevents mangiferin MPT-eliciting capacity and enables it to prevent MPT apparently by ROS scavenging and protection of mitochondrial membrane protein thiols and glutathione from oxidation.

Materials and Methods

Reagents and Solutions. Mangiferin, ferric chloride anhydrous, and the other reagents were purchase from Sigma (St. Louis, MO). Ferric ($FeCl_3$ + 10 mM HCl) ion solutions were prepared in Milli-Q water and used immediately. Stock solutions of mangiferin in dimethyl sulfoxide were used with dilutions of 1/1000 in assay medium to minimize solvent interference. The mangiferin/Fe(III) complex (2:1) was prepared by mixing 97.5-μl stocks solutions of 25 mM mangiferin and 12.5 μl of 100 mM $FeCl_3$. The mixture was preincubated for 10 min before each experiment and used daily with dilutions of 1/1000 in assay medium. EDTA (50 μM) was used in some experiments to release ferric ions from mangiferin. In such cases, EDTA was preincubated for 10 min with the mangiferin-Fe(III) complex and used with a dilution of 1/1000. All experiments were performed at 30°C.

Electrochemical Assays. Electrochemical assays were carried out with a BAS CV-27 potentiostat (BAS Bioanalytical Systems, West Lafayette, IN) and recorded on an Omnigraphic XY recorder (Houston Instruments, Houston, TX). Conventional electrochemical cells with three electrodes were employed. A glassy carbon electrode (geometric area = 0.0314 cm^2) was used as the working electrode. The electrode was polished before use with a 1-μ alumina water suspension and rinsed thoroughly with water and acetone. A platinum wire was used as a counter electrode, and all potentials are referenced to a sodium-saturated silver/silver chloride electrode [$Ag/AgCl/NaCl_{(sat)}$], irrespective of the liquid junction potential. Cyclic voltametric studies were carried out at sweep rates of 100 mV/s in 5 ml of phosphate buffer, pH 7.2, in which 50 μl of 100 mM dimethyl sulfoxide solution of mangiferin was added. Because the cyclic voltammograms were recorded at a window potential including negative ranges (−0.4 to 1.0 V), oxygen-free solution was obtained by bubbling argon through it. When the potential was scanned, the inert gas was kept in the solution to ensure that any oxidation reaction was not initiated by oxygen. For the electrochemical oxidation of mangiferin in presence of Fe(III), aliquots of the acidic aqueous fresh solution of $FeCl_3 \cdot 6H_2O$ were added to give the final concentrations. The solution was stirred for 30 s and allowed to rest for 30 s for equilibration. The potential was scanned at 100 mV/s.

UV-Visible Spectroscopical Analysis. The absorption spectra between 300 to 700 nm of mangiferin were recorded with a Shimadzu UV-visible spectrophotometer V-240 (Shimadzu, Kyoto, Japan) under the same conditions as in the electrochemical experiments. Aliquots of the acidic aqueous solution of $FeCl_3 \cdot 6H_2O$ were added to give the final concentrations shown.

Isolation of Rat Liver Mitochondria. Mitochondria were isolated by standard differential centrifugation. Male Wistar rats weighing approximately 200 g were sacrificed by cervical dislocation. The livers (10–15 g) were immediately removed, sliced in 50 ml of medium containing 250 mM sucrose, 1 mM EGTA, and 10 mM HEPES-KOH, pH 7.2, and homogenized three times for 15 s at 1-min intervals with a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 580*g* for 5 min, and the resulting supernatant was further centrifuged at 10,300*g* for 10 min. Pellets were suspended in 10 ml of medium containing 250 mM sucrose, 0.3 mM EGTA, and 10 mM HEPES-KOH, pH 7.2, and centrifuged at 3400*g* for 15 min. The final mitochondrial pellet was suspended in 1 ml of medium containing 250 mM sucrose and 10 mM HEPES-KOH, pH 7.2, and used within 3 h. Mitochondrial protein content was determined by the biuret reaction. The respiratory control ratio of mitochondria was always in the 4.5 to 6.0 range.

Estimation of Mitochondrial Swelling. Swelling of mitochondria was estimated from the decrease in apparent absorbance at 540 nm, using a model DU-70 Beckman spectrophotometer (Beckman Coulter, Fullerton, CA), in a standard incubation medium containing 125 mM sucrose, 65 mM KCl, and 10 mM HEPES-KOH, pH 7.2.

Assessment of H_2O_2 Production. Generation of ROS by mitochondria was monitored using the Amplex Red assay for H_2O_2 (Molecular Probes, Eugene, OR) (Votyakova and Reynolds, 2001). Mitochondria (0.5 mg/ml) were incubated in the standard medium described above, supplemented with 10 μM Amplex Red, 1 U/ml horseradish peroxidase, and 1 μM antimycin A at 30°C with continuous stirring. Fluorescence of oxidized probe was measured in a Hitachi F-4010 spectrofluorometer at 563 nm for excitation and 587 nm for emission (Hitachi, Tokyo, Japan). Standard curves obtained by adding known amounts of H_2O_2 to assay medium in the presence of the reactants (Amplex Red and horseradish peroxidase) were linear up to 2 μM. The slope of the increase in fluorescence was converted to the rate of H_2O_2 production (10 fluorescence units represent 82 pmol H_2O_2). As expected, the addition of catalase (400 U/ml) decreased the fluorescence by 85 to 90% (data not shown).

Estimation of Mitochondrial Lipid Peroxidation. Lipid peroxidation was estimated from malonyldialdehyde (MDA) generation (Buege and Aust, 1978). The mitochondrial suspension (1 ml, 1 mg of protein) was incubated in the standard medium with 300 μM *t*-butyl hydroperoxide at 37°C. After 20 min, 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 were added, followed by incubation for 20 min at 85°C. The MDA-thiobarbituric acid complex was extracted with 2 ml of *n*-butanol, and absorbance was measured at 535 nm. MDA concentration was calculated from $\varepsilon = 1.56 \times 10^5$ M/cm.

Determination of 1,1-Diphenyl-2-Picrylhydrazyl Radical Scavenging Activity. Scavenging activity of the compounds against 100 μM 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was monitored by the change in absorbance at 517 nm, 5 min after incubation in 40 mM sodium acetate, pH 5.5, and 1 ml of ethanol (Blois, 1958).

Determination of Mitochondrial Glutathione Content. 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 the fluorescence was measured 15 min later using the 350-/420-nm excitation/emission wavelength pair and a F-4500 fluorescence spectrophotometer (Hitachi) (Hissin and Hilf, 1976).

Determination of Protein Thiol Group Content. Mitochondrial membrane thiol groups were determined using 5,5′-dithiobis(2-nitrobenzoic)acid (Ellman's reagent) as described previously (Kowaltowski and Castilho, 1997). The mitochondrial suspension was incubated briefly in standard reaction medium, submitted to three subsequent freeze-thawing procedures to release matrix proteins, and then centrifuged for 2 min at 6708*g* in a 5415 C Eppendorf Centrifuge (Eppendorf-5 Prime, Inc., Boulder, CO). The pellet was treated with 200 μl of 6.5% trichloroacetic acid and centrifuged at 6708*g* for 2 min to precipitate the protein. This procedure was repeated twice. 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.

Statistical Analysis. The results are presented as the mean ± S.D., and statistical significance between the groups was deter-

mined by means of one-way analysis of variance followed by one-tailed Student's t test to determine statistical significance. p values lower than 0.05 were considered indicative of significance.

Results

Fe(III) Coordinates with Mangiferin, as Evidenced by UV-Visible Spectroscopical and Electrochemical Analyses. Figure 1A shows a characteristic UV-visible spectrum of mangiferin in 100 mM phosphate buffer, at pH 7.2. The addition of Fe(III) induced a dose-dependent red shift in the position of the maximal absorption of mangiferin (from 379 to 387 nm). The occurrence of a new family of spectra originating from mangiferin was characterized by the presence of an isosbestic point close to 363 nm with absorption peaks emerging at 470 nm. The broad bands at 470 nm together with the isosbestic point, suggest the formation of a ligand with a metal charge transfer to a mangiferin complex. This is confirmed by the observation that the original mangiferin spectrum was fully recovered following treatment with the iron chelator EDTA.

Figure 1B shows the voltametric behavior of 1 mM mangiferin. An irreversible oxidation at approximately +0.65 mV versus Ag/AgCl/NaCl saturated, as shown by the results of the first anodic scan, decreased in amplitude after subsequent scanning. We ascribe this effect to a catechol-localized process in mangiferin (Danilewicz, 2003). Fe(III) (up 0.5 mM) promoted a concentration-dependent reduction of the anodic current's peak height, apparently resulting from coordination of the catechol moiety of mangiferin with iron at a 2:1 ligand/metal stoichiometry. At a higher iron concentration (2 mM), a change in the linear reduction of the anodic current was observed, probably due to coordination of the excess metal to the adjacent 4-carbonyl/1-hydroxy groups. Interestingly, the iron coordination by mangiferin shifts its oxidation potential to more cathodic values (0.65–0.6 mV), reflecting the increase in the reducing capacity of the complex compared with that of mangiferin alone.

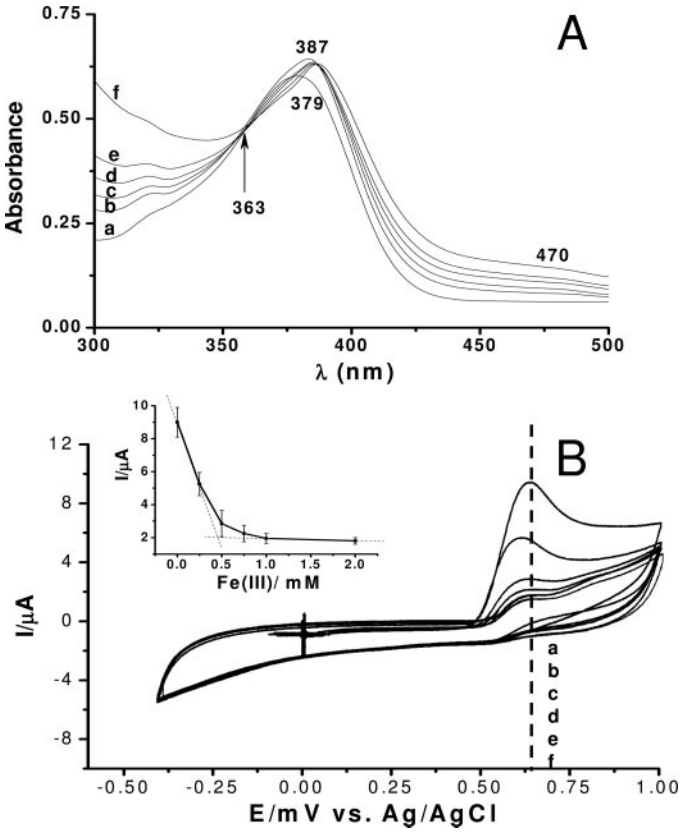

Fig. 1. Chemical evidences of mangiferin-Fe(III) complex formation. A, UV-visible absorption spectra of 20 μM mangiferin in 0.1 M phosphate buffer, pH 7.2, in the absence (a) or presence (2 min of incubation before spectrum acquisition) of Fe(III) at: 5 (b), 10 (c), 15 (d), 20 (e), and 40 (f) μM. The addition of EDTA to (f) fully recovered the spectrum (a). B, cyclic voltammograms of 1 mM mangiferin in 0.1 M phosphate buffer, pH 7.2, in the absence (a) or presence of Fe(III) at: 0.25 (b), 0.5 (c), 0.75 (d), 1 (e), and 2 (f) mM. The scan rate was 100 mV/s, using a glassy carbon electrode (area = 0.0314 cm^2). Typical examples are shown. For experimental details, see *Materials and Methods*.

In addition, using spectroscopy analysis, we have provided the following evidence. More than 2 mol of mangiferin bind to 1 mol of Fe(III) (data not shown, determined by the Job's method of continuous variation according to Cartwright, 1986), which means that the mixture of mangiferin and Fe(III) at 2:1 ratio will render the (mangiferin)$_2$-Fe(III) complex as the most abundant specie in solution, and albumin (0.6 mM), which is an important nonspecific plasma complexant protein, causes only small changes in the spectrum of mangiferin or mangiferin-Fe(III) 2:1, suggesting that the interaction of iron or mangiferin with this plasma protein is not so favored (data not shown).

Mangiferin-Fe(III) Complex Does Not Elicit MPT. We demonstrated previously that mangiferin elicits MPT in succinate-energized isolated rat liver mitochondria exposed to Ca^{2+} (Andreu et al., 2005a; Pardo-Andreu et al., 2006b). Here, we confirm this result (Fig. 2) by observing significant Ca^{2+}-dependent, dithiothreitol- and CsA-sensitive mitochon-

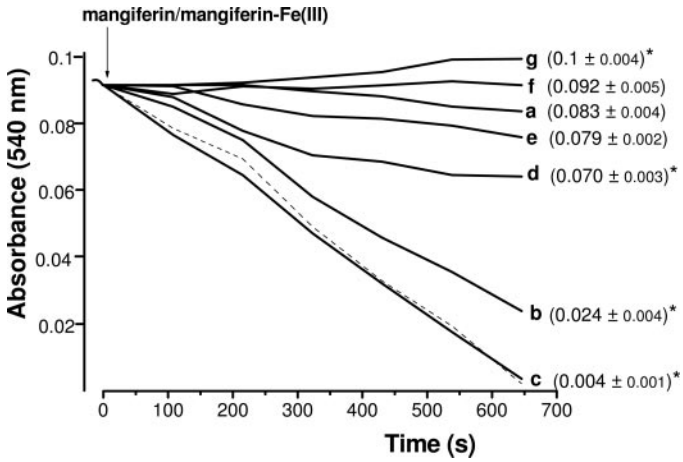

Fig. 2. Swelling-inducing capacity of mangiferin and mangiferin-Fe(III) (2:1) on isolated rat liver mitochondria (0.4 mg of protein) suspended at 30°C in a standard medium containing 125 mM sucrose, 65 mM KCl, 5 mM potassium succinate, 2.5 μM rotenone, 10 μM Ca^{2+}, and 10 mM HEPES-KOH, pH 7.4, to a final volume of 1 ml. a, no mangiferin or mangiferin-Fe(III) addition; b, 25 μM mangiferin; c, 50 μM mangiferin; d, 25 μM mangiferin-Fe(III); e, 50 μM mangiferin-Fe(III); f, 50 μM mangiferin plus 1 μM cyclosporin A; g, 50 μM mangiferin plus 500 μM dithiothreitol. The dashed line represents an experiment conducted under the conditions of e, plus 50 μM EDTA. Fe(III) (50 μM) did not elicit swelling after 10-min incubation with mitochondria. The tracings are representative of three experiments using different mitochondrial preparations. Absorbance values after 10-min incubation were obtained from these traces and presented as mean ± S.D. in the figure caption. One-tailed Student's t test was used for statistical analysis. *, $p < 0.05$ comparing condition a with the other conditions.

drial swelling under these conditions. However, no significant swelling was induced by 25 or 50 μM mangiferin in the presence of Fe(III) (2:1 mangiferin:iron ratio). Furthermore, EDTA significantly prevented the effect of Fe(III) addition. This result indicates that, in the presence of Fe(III), mangiferin does not elicit MPT.

Mangiferin-Fe(III) Complex Prevents MPT. Figure 3A shows swelling induced by Ca^{2+} plus phosphate in succinate-energized mitochondria. Mangiferin alone stimulated this swelling on account of its intrinsic ability to elicit MPT (see above). In the presence of Fe(III), however, swelling was significantly inhibited. The addition of EDTA significantly prevented the inhibition. Figure 3, B and C, shows the effects of mangiferin, in the absence or presence of Fe(III), on swelling induced by 300 μM *t*-butyl hydroperoxide in succinate-energized mitochondria in the presence of 10 μM Ca^{2+} or nonenergized organelles in the presence of 500 μM Ca^{2+}, a condition that rules out significant interference of mitochondrial respiration or inner membrane potentials. The results closely followed those of Fig. 3A and together indicate that, in the presence of Fe(III), mangiferin prevents MPT.

Mangiferin-Fe(III) Complex Scavenges Mitochondrial ROS. We evaluated whether scavenging of mitochondria-generated ROS would be involved in the MPT-preventing capacity of mangiferin-Fe(III) by assessing H_2O_2 production (Fig. 4A) and membrane lipid peroxidation (Fig. 4B) in mitochondria exposed to 1 μM antimycin A or 300 μM *t*-butyl hydroperoxide, respectively. Oxidizing succinate in absence of respiratory chain inhibitors, the production of H_2O_2 by mitochondria was low. As expected (Sugioka et al., 1988), inhibition of the respiratory chain complex III by antimycin A markedly increased H_2O_2 production (Fig. 4A). Mangiferin alone lowered H_2O_2 production by almost 50%, but in the presence of Fe(III), this antioxidant effect was increased dose-dependently, allowing 100% lowering in the presence of the complex (50 μM). Mitochondrial membrane lipid peroxidation induced by 20-min incubation of mitochondria with 300 μM *t*-butyl hydroperoxide was assessed by MDA formation (Fig. 4B). The profile for the inhibition of this process by mangiferin, in the absence or presence of Fe(III), closely followed the profile for inhibition of H_2O_2 production, described above. Again, EDTA significantly prevented these Fe(III) effects. Furthermore, Fig. 5 shows that, in the presence of Fe(III), mangiferin has an approximately 2-fold higher capacity to scavenge the DPPH radical. Together, these results indicate that Fe(III) improves the scavenging activity of mangiferin toward ROS, including that on mitochondria, in agreement with our recent findings on superoxide scavenging and cytoprotection (Pardo-Andreu et al., 2006c). Therefore, scavenging of mitochondria-generated ROS is likely to contribute for the MPT-preventing capacity of the mangiferin-Fe(III) complex.

Mangiferin-Fe(III) Complex Protects against Mitochondrial Glutathione and Membrane Protein Thiol Oxidation. Because MPT is intimately associated with glutathione (GSH) and mitochondrial protein thiol redox state (Kowaltowski et al., 2001), we evaluated whether mangiferin alone or in the presence of Fe(III) could alter this mitochondrial thiol redox status (Fig. 6). A direct relationship was observed between these effects and mitochondrial swelling (Figs. 2 and 3), i.e., the pattern of swelling induction by mangiferin followed the pattern of oxidation of mitochondrial GSH and membrane protein thiols. Furthermore, the pattern

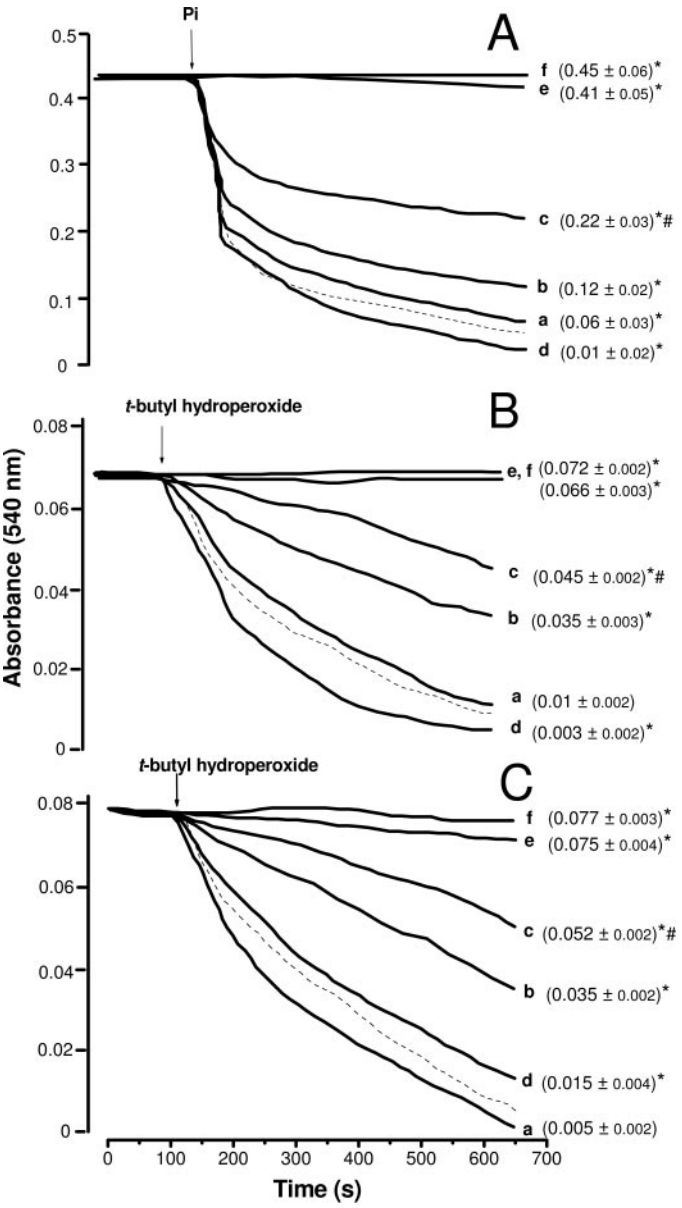

Fig. 3. Swelling inhibiting capacity of mangiferin and mangiferin-Fe(III). Effects of mangiferin and mangiferin-Fe(III) complex (2:1) on swelling elicited by 10 μM Ca^{2+} plus 1 mM inorganic phosphate (P_i) (A) or 10 μM Ca^{2+} plus 300 μM *t*-butyl hydroperoxide (B) on isolated rat liver mitochondria energized by 5 mM potassium succinate (+2.5 μM rotenone) or by 500 μM Ca^{2+} plus 300 μM *t*-butyl hydroperoxide in nonenergized organelles (C). Mitochondria (0.4 mg of protein) were incubated at 30°C with the compounds in a standard medium containing 125 mM sucrose, 65 mM KCl, and 10 mM HEPES-KOH, pH 7.4, in the presence of succinate/rotenone and/or Ca^{2+}, in a final volume of 1 ml. a, absence of mangiferin or mangiferin-Fe(III); b, 25 μM mangiferin-Fe(III); c, 50 μM mangiferin-Fe(III); d, 50 μM mangiferin; e, absence of Ca^{2+}; f, condition d plus 1 μM CsA. Fe(III) (50 μM) did not elicit or prevent swelling after 10-min incubation with mitochondria. The dashed lines represent experiments conducted under the conditions in c plus 50 μM EDTA. The tracings are representative of three experiments using different mitochondrial preparations. Absorbance values after 10-min incubation were obtained from these traces and presented as mean ± S.D., in the figure caption. One-tailed Student's *t* test was used for statistical analysis. *, $p < 0.05$ comparing condition a with the other conditions. #, $p < 0.05$ comparing conditions b versus c.

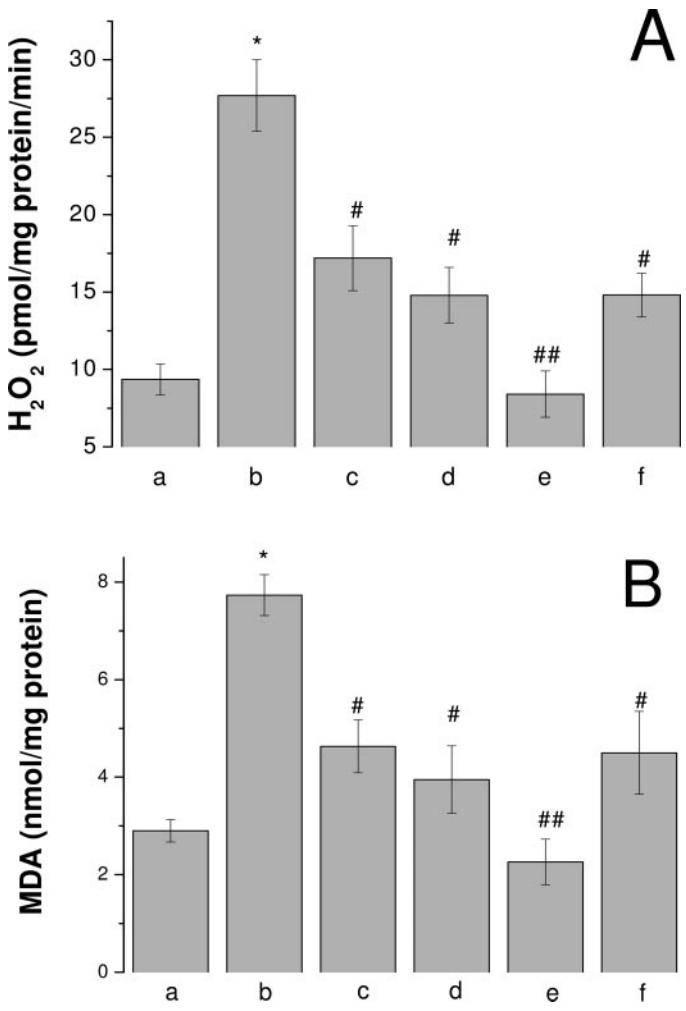

Fig. 4. Antioxidant activity of mangiferin and mangiferin-Fe(III) on mitochondria. Effects of mangiferin and mangiferin-Fe(III) complex (2:1) on 1 μM antimycin A-induced H_2O_2 production on isolated rat liver mitochondria (0.5 mg of protein) (A) or 300 μM *t*-butyl hydroperoxide-induced lipid peroxidation, assessed as MDA, on isolated rat liver mitochondria (1 mg of protein), incubated at 30°C in a standard medium containing 125 mM sucrose, 65 mM KCl, 5 mM potassium succinate, and 10 mM HEPES-KOH, pH 7.4, to a final volume of 2 ml (B). a, no antimycin or *t*-butyl hydroperoxide; b, 1 μM antimycin A or 300 μM *t*-butyl hydroperoxide; c, the condition b plus 50 μM mangiferin; d, the condition b plus 25 μM mangiferin-Fe(III); e, the condition b plus 50 μM mangiferin-Fe(III); f, 50 μM mangiferin-Fe(III) plus 50 μM EDTA. Fe(III) (25 μM) alone did not affect H_2O_2 production in b, although it increased MDA formation when it was added together with *t*-butyl hydroperoxide (data not shown). *, $p < 0.05$ compared with a; #, $p < 0.05$ compared with b; ##, $p < 0.05$ compared with d.

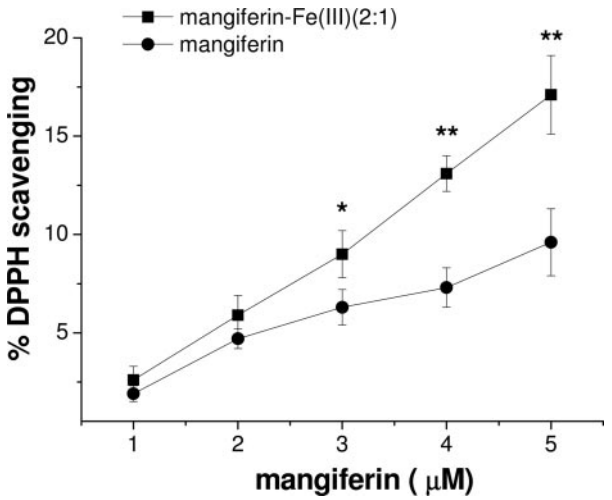

Fig. 5. Scavenging activity of mangiferin and mangiferin-Fe(III). Effects of mangiferin and mangiferin-Fe(III) complex (2:1) on DPPH scavenging, as described under *Materials and Methods*. Values are means ± S.D. of the results of three different experiments and relative to a control in absence of mangiferin and in the presence of 14.3 μM dimethyl sulfoxide, the solvent for mangiferin. $A_{517\ nm}$ value for control was 0.422 ± 0.001. One-tailed Student's *t* test was used for statistical analysis; *, $p < 0.05$; **, $p < 0.01$.

of swelling inhibition by mangiferin-Fe(III) closely followed the pattern of inhibition of the oxidation of mitochondrial GSH and membrane protein thiols by the complex. These results suggest that the protection of mitochondrial membrane protein thiols and/or GSH from oxidation accounts for the MPT-preventing capacity of the mangiferin-Fe(III) complex.

Discussion

The present study demonstrates that coordination of mangiferin with Fe(III) prevents the MPT-eliciting capacity previously reported for the compound (Andreu et al., 2005a; Pardo-Andreu et al., 2006b) and enables it to prevent MPT elicited by Ca^{2+} plus phosphate or by Ca^{2+} plus *t*-butyl hydroperoxide, both in energized and nonenergized mitochondria. Although protection of mangiferin's cathechol moiety by hindering the formation of metabolites able to mediate arylation of membrane protein thiols probably accounts for the former effect, increase of the scavenging activity of mangiferin toward ROS probably accounts for the latter. Therefore, Fe(III) prevents the prooxidant action of mangiferin on mitochondria concomitantly with stimulation of its antioxidant activity. As far as MPT is concerned, this dual effect of iron, demonstrated here for the first time, could constitute a potential protective mechanism for the prooxidant actions of mangiferin and other polyphenol-like catechol-containing antioxidants.

The structure of mangiferin—a nearly planar dibenzo-γ-pyrone ring with an extensive π-conjugated system—strongly favors iron chelation, and the catechol moiety (6,7-hydroxy groups at B ring), which dissociates at pH 7.2, is probably involved in this property. It is conceivable that as consequence of catechol-Fe(III) coordination, mangiferin acquires an additional effective superoxide dismuting metal center, which increases its reducing power. Furthermore, such a metal center could protect the catechol moiety of mangiferin from oxidation by ROS, slowing down mangiferin quinone derivative formation, thus preventing oxidation of mitochondrial thiol groups (GSH plus membrane protein thiols) and, consequently, MPT. Accordingly, metal complexes with catechol antioxidants have been reported to undergo electron transfer reactions comparable with those known for superoxide dismutase (Kostyuk et al., 2004), and iron complexation by catechol-like flavonoids, in particular, has been reported to hinder the oxidation of the ligand despite the increase in their antioxidant capacity (Kostyuk et al., 2004; Mahal et al., 2005).

Figure 7 shows a proposed mechanism for the effects of mangiferin/mangiferin-Fe(III) on MPT induction/inhibition. Relatively high Ca^{2+} levels in mitochondria, or other condi-

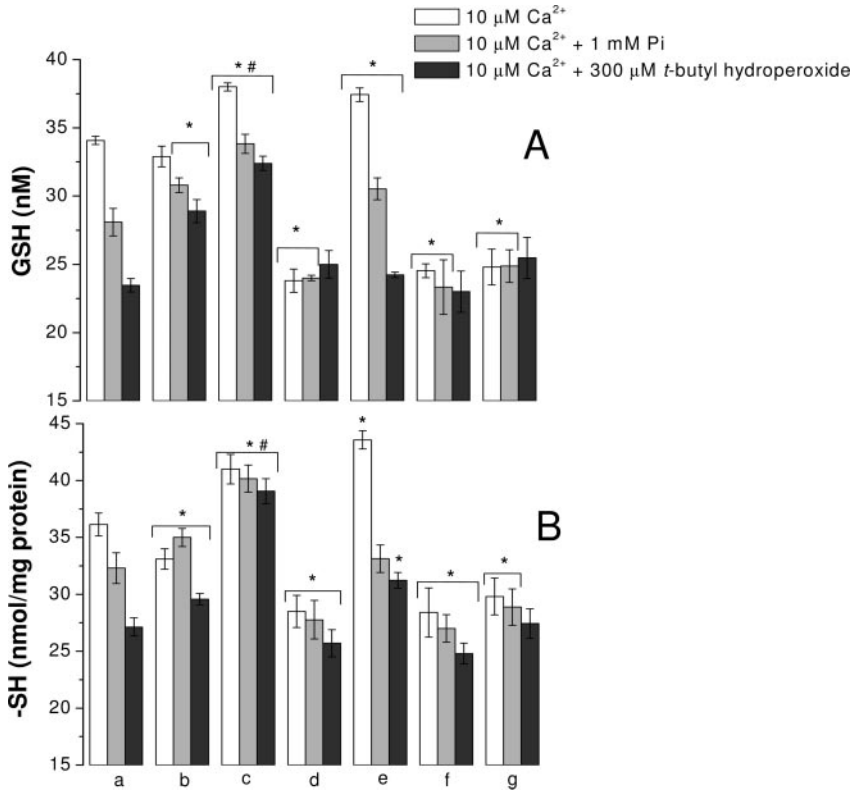

Fig. 6. Effects of mangiferin and mangiferin-Fe(III) on mitochondrial GSH (A) and membrane protein thiols (B) oxidations. Rat liver mitochondria were incubated 10 min under the same MPT experimental conditions described in the legends of Fig. 2 (MPT induced by 10 μM Ca^{2+}; white bars), Fig. 3A (MPT induced by 10 μM Ca^{2+} + 1 mM; light gray bars), and Fig. 3B (MPT induced by 10 μM Ca^{2+} + 300 μM *t*-butyl hydroperoxide; dark gray bars). GSH (1 mg/ml mitochondrial protein) and membrane protein thiols (0.5 mg/ml) were determined as described under *Materials and Methods*, interpolating the absorbance values in a calibration curve with standard GSH. The results represent means ± S.D. (n = 3 independent mitochondrial preparations). The experimental conditions were: a, no mangiferin or mangiferin-Fe(III) addition; b, 25 μM mangiferin-Fe(III(; c, 50 μM mangiferin-Fe(III); d, 50 μM mangiferin; e, absence of Ca^{2+}; f, 50 μM mangiferin plus 1 μM cyclosporin A; g, an experiment conducted under the conditions of e plus 50 μM EDTA. Fe(III) (50 μM) alone did not affect either mitochondrial GSH or protein thiol groups content. One-tailed Student's t test was used for statistical analysis. *, $p < 0.05$ comparing condition a versus the other conditions; #, $p < 0.05$ comparing conditions b versus c.

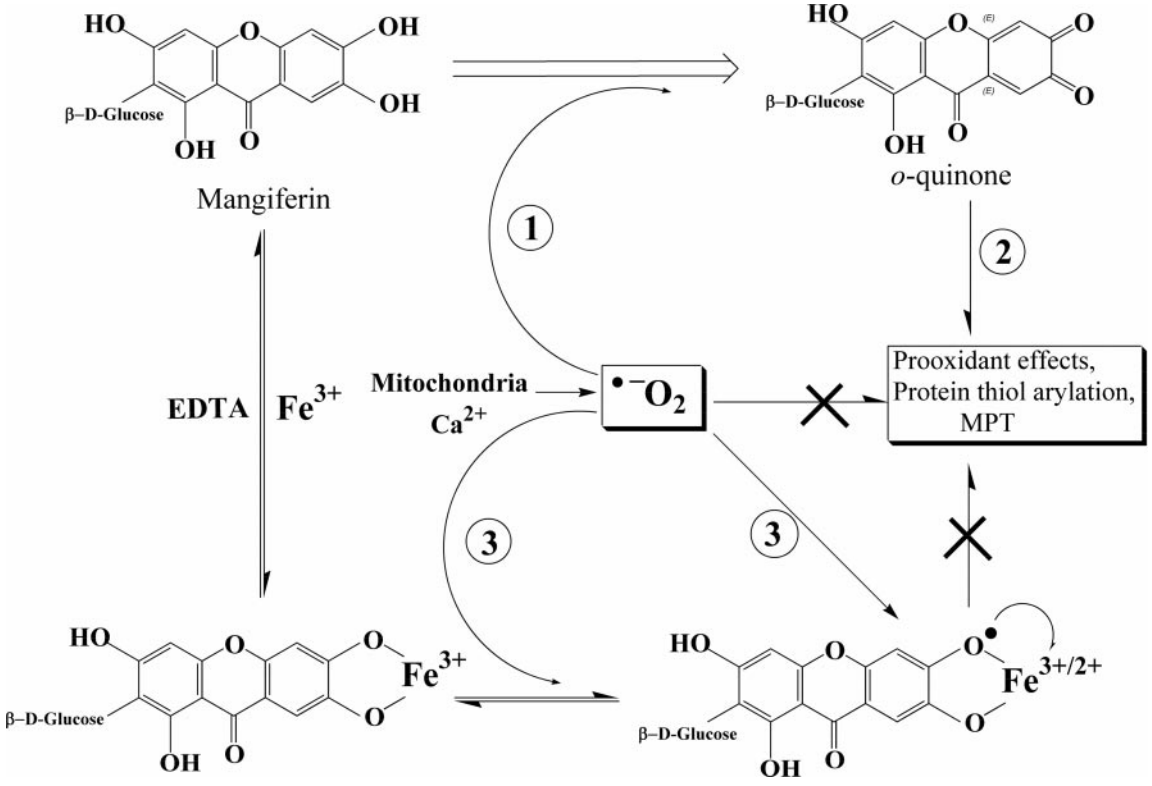

Fig. 7. Proposed mechanism for the effects of mangiferin/mangiferin-Fe(III) on MPT induction/inhibition. Relatively high Ca^{2+} levels in mitochondria, or other conditions causing oxidative stress, lead to increase in superoxide radicals, which are scavenged by the mangiferin's catechol moiety. Mangiferin becomes oxidized, accumulating quinone/quinone methide metabolites (1, antioxidant route), that have the capacity to arylate mitochondrial protein thiols (2, prooxidant route). Fe(III) coordination by the catechol moiety of compound, besides increasing its scavenging activity toward ROS (3), decreases the availability of the quinone metabolites able to interact with mitochondrial protein thiols. Although the former effect prevents MPT elicited by Ca^{2+} plus ROS, the latter prevents MPT induction by mangiferin itself. Mangiferin-Fe(III) at 1:1 ratio was presented for simplicity. See text for details.

tions causing oxidative stress, lead to increase in superoxide radicals, which are scavenged by the mangiferin's catechol moiety. Mangiferin becomes oxidized, accumulating quinone/quinone methide metabolites (1, antioxidant route) that have the capacity to arylate mitochondrial protein thiols (2, prooxidant route). This mechanism has recently been proposed by us for the MPT-inducing property of mangiferin (Andreu et al., 2005a). Fe(III) coordination by the catechol moiety of compound, besides increasing its scavenging activity toward ROS (3), decreases the availability of the quinone metabolites able to interact with mitochondrial protein thiols. Although the former effect prevents MPT elicited by Ca^{2+} plus ROS, the latter prevents MPT induction by mangiferin itself, both via the attenuation of the oxidation of catechol to quinone metabolites and via the decrease in availability of the superoxide ready to produce the quinone metabolites from mangiferin alone. The protection of mangiferin-Fe(III) complex observed against mitochondrial GSH and membrane protein thiol oxidation strongly supports this model.

A direct chelation of mitochondrial iron has been recently proposed as an attractive strategy for managing a number of clinical disorders involving iron imbalance (Huang et al., 2006). In this sense, the modulation by iron of both antioxidant and prooxidant actions of mangiferin regarding MPT, and possibly of other catechol-containing polyphenols (e.g., 3′,4′-dihydroxy flavonoids), may have potential therapeutic relevance. Iron overloading in animals has been reported to cause dissipation of membrane potential and increase of calcium efflux in mitochondria (Masini et al., 2000; Pietrangelo et al., 2002; Rauen et al., 2004), processes that are often associated with MPT. Furthermore, MPT itself may be elicited or facilitated by iron (Hermes-Lima et al., 1995; Gogvadze et al., 2003). Because mangiferin appears to reach mitochondria after oral administration to rats, eliciting either antioxidant or prooxidant action on the organelles (Andreu et al., 2005a; Pardo-Andreu et al., 2006b), it is conceivable that iron-loaded mitochondria may constitute potential therapeutic targets for the compound, which could decrease the levels of iron via chelation, with consequent improvement of its antioxidant performance against MPT. Conversely, during cancer progression, the cellular level of iron decreases (Benhar et al., 2002; Torti and Torti, 2002), a condition that is associated with an aggressive malignant phenotype (Torti and Torti, 2002; Pham et al., 2004). This low iron environment would allow mangiferin to directly scavenge ROS and generate quinone-like metabolites eliciting MPT, which in turn would trigger cell death by necrosis or apoptosis. We therefore envisage for mangiferin and perhaps for other cathechol-containing polyphenols a potential use either in preventing or eliciting cell death, simply in connection with the availability of free/labile-bound iron in the cell environment. These circumstances should be taken into account during human polyphenol supplementation, for either the prevention or not of their prooxidant effects, as well as for optimization of their antioxidant performance.

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