Curcumin induces the mitochondrial permeability transition pore mediated by membrane protein thiol oxidation

Didier Morin[a,b,*], Sophie Barthélémy[c], Roland Zini[a], Serge Labidalle[c], Jean-Paul Tillement[a]

[a] *Laboratoire de Pharmacologie, Faculté de Médecine, Créteil, France*
[b] *CNRS, Faculté de Médecine, Créteil, France*
[c] *Faculté de Pharmacie, Toulouse, France*

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Abstract Curcumin is a natural compound showing antiproliferative properties. Recent studies suggest that these properties might be due to its ability to induce apoptosis in tumor cells. As mitochondria play a pivotal role in the induction of the apoptotic process, we analyzed the effect of curcumin on mitochondrial function. Curcumin induced an increase in rat liver mitochondrial membrane permeability, resulting in swelling, loss of membrane potential and inhibition of ATP synthesis. These effects were mediated by the opening of the permeability transition pore. Curcumin pore induction involved the oxidation of membrane thiol functions and required the presence of low Ca^{2+} concentrations. These data suggest that mitochondria might be a target by which curcumin induces apoptosis of tumor cells.

Key words: Curcumin; Mitochondrial membrane potential; Calcium flux; Thiol group; Oxidation

1. Introduction

Curcumin (CUR; 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a natural yellow pigment isolated from the rhizome of *Curcuma longa* L. (turmeric). It possesses a wide range of pharmacological properties including antiinflammatory, hypocholesterolemic and antiinfectious activities [1–3].

CUR has also been shown to display anticarcinogenesis properties in a wide variety of cell lines [4,5] and in animals [6,7]. Recently, it was suggested that the effect of CUR could be mediated through its ability to induce apoptosis of tumor cells [8,9]. Similar results were found in osteoclasts [10] and in thymocytes, but the features of the apoptotic cell death induced by CUR appeared different from those observed with the typical proapoptotic agent dexamethasone [11]. Growing evidence suggests that mitochondria are involved in the induction of the cell death program [12]. Numerous stimuli can trigger an increase in mitochondrial membrane permeability [13] which is due to the formation of a pore, called the permeability transition pore (PTP), at the level of the contact sites between the inner and the outer mitochondrial membranes. PTP opening induces the collapse of the mitochondrial membrane potential ($\Delta\psi$), respiration impairment and the inhibition of ATP synthesis. It releases apoptogenic factors and can lead to cell death [14]. Therefore, novel antitumor strategies now focus on mitochondria. A recent study suggested that the apoptotic process mediated by CUR in AK-5 tumor cells involved a hyperproduction of radical oxygen species (ROS) and a loss of $\Delta\psi$ [15]. In the present study, we examined the effect of CUR on isolated liver mitochondria and found that CUR is able to induce PTP opening.

2. Materials and methods

Rat liver mitochondria were isolated from male Wistar rats as described previously [16]. Mitochondrial swelling was assessed by measuring the change in absorbance of their suspension at 520 nm by using a Hitachi® model U-3000 spectrophotometer either in energized or deenergized conditions as described by Elimadi et al. [16]. $\Delta\psi$ was monitored by means of the fluorescent dye rhodamine 123 at the excitation and emission wavelengths of 503 and 527 nm, respectively [16]. Ca^{2+} flux and mitochondrial respiration were simultaneously measured in a thermostat-controlled reaction chamber (4 ml) at 25°C. Mitochondrial respiration was measured by means of a Clark-type oxygen microelectrode fitted to an oxygen monitoring system (Hansatech®). The concentration of Ca^{2+} in the extramitochondrial medium was monitored using a specific Ca^{2+} electrode (Orion®) connected to the auxiliary output of the oxygen monitoring system via a 720 A Orion ionometer.

Lipid peroxidation was assayed as the generation of thiobarbituric acid-reactive substances (TBARS) according to [17] and the generation of $O_2^{\bullet -}$ was achieved as previously reported [17]. For this particular experiment, mitochondria were purified on a sucrose gradient [18] to avoid microsome contamination. Cyclosporin A (CsA) was added to the medium to inhibit mitochondrial swelling, which slightly interfered with the spectroscopic detection of the reduction reaction.

Protein thiol content was measured according to [19] with some modifications. Briefly, mitochondria (1 mg/ml) were incubated in the respiration buffer in the presence of CUR or other agents in a total volume of 1 ml for 15 min at 25°C. After this time, 100 μl of mitochondrial solution were added to 700 μl of methanol and 200 μl of Tris–EDTA (250/20 mM; pH = 8.2 at 20°C). 20 μl of Ellman's reagent (10 mM) were then added and the reaction was incubated for 15 min at room temperature. Absorbance of the medium was read at 410 nm against an Ellman's reagent blank. In order to estimate protein thiol concentration, a glutathione concentration range was performed under the same conditions.

*Corresponding author. Département de Pharmacologie, Faculté de Médecine de Paris XII, 8 rue du Général Sarrail, F-94010 Créteil, France. Fax: (33)-1-49 81 35 94.
E-mail: morin@univ-paris12.fr

Abbreviations: CUR, curcumin; CsA, cyclosporin A; t-BH, *tert*-butylhydroperoxide; NEM, *N*-ethylmaleimide; BHT, 2,6-di-*tert*-butyl-4-methylphenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; RCR, respiratory control ratio; PTP, permeability transition pore; $\Delta\psi$, mitochondrial membrane potential; TBARS, thiobarbituric acid-reactive substances; Tris, 2-amino-2-hydroxymethyl-propan-1,3-diol; ROS, reactive oxygen species

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Curcumin (purity >99%) was synthesized by Synthe-Pharma (Seysses, France).

3. Results and discussion

3.1. Curcumin induces mitochondrial swelling

In the experiment depicted in Fig. 1A, rat liver mitochondria energized with succinate were incubated for 1 min in a sucrose phosphate buffer before the addition of increasing concentrations of CUR. The light scattering of the mitochondrial suspension was then monitored. CUR induced mitochondrial swelling as attested by the decrease in absorbance in a concentration-dependent manner; the maximal effect was obtained at 20 μM. This effect was completely inhibited in the presence of 1 μM CsA or 150 μM 2,6-di-*tert*-butyl-4-methylphenol (BHT; Fig. 1B). Thus, CUR-induced mitochondrial swelling is due to the opening of the CsA-dependent pore [13,20]. This is confirmed by the fact that ubiquinone 0, which has recently been shown to inhibit PTP opening [21], also reversed CUR-induced swelling (Fig. 1B).

PTP opening was also assessed by measuring $\Delta\psi$ and Ca^{2+} flux. As shown in Fig. 2, 20 μM CUR collapsed $\Delta\psi$ and induced Ca^{2+} release. These effects were prevented by 1 μM CsA. These results are in agreement with the data of Jaruga et al. [11], who observed a rapid collapse of $\Delta\psi$ after addition of CUR to thymocytes. Under the same experimental conditions, 10 μM of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which collapses $\Delta\psi$ and releases Ca^{2+} (Fig. 2), was unable to induce swelling (Fig. 1B).

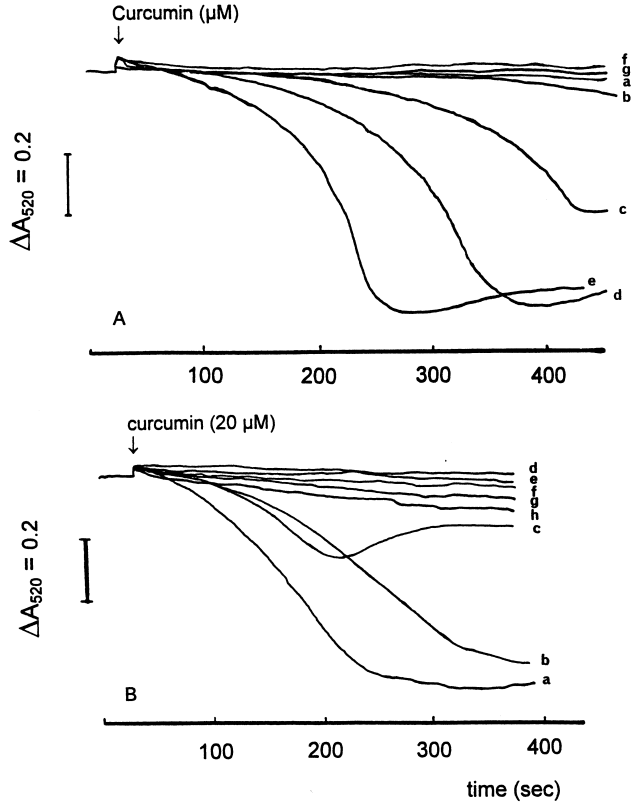

Fig. 1. Induction of mitochondrial swelling by curcumin (CUR). Liver mitochondria were preincubated for 3 min in a buffer containing 0.25 M sucrose, 5 mM KH_2PO_4, and 6 mM succinate, pH = 7.2 at 25°C. A: Induction of swelling by increasing concentrations of curcumin, 2.5 μM (line b), 10 μM (line c), 15 μM (line d), 20 μM (line e). Line a: No addition of CUR. Lines f and g: Mitochondria were preincubated with 1 μM CsA (f) or 150 μM BHT (g) before swelling induction. B: Swelling was induced by 20 μM CUR (line a), in the presence of 2 μM rotenone (line b) and pyruvate/malate (6/6 mM) instead of succinate (line c). Preincubation (3 min) of mitochondria with either 10 μM CCCP (line d), 20 μM EGTA (line e), 1 μM ruthenium red (line f), 20 μM NEM (line g) or 50 μM ubiquinone 0 (line h) counteracted the effect of CUR.

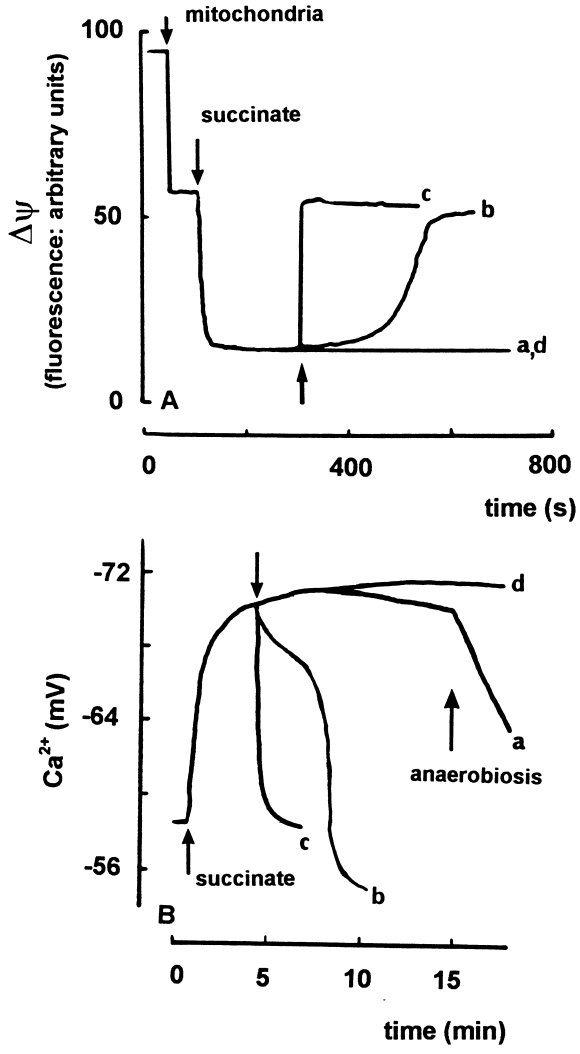

Fig. 2. Effect of curcumin (CUR) on mitochondrial membrane potential ($\Delta\psi$) and Ca^{2+} fluxes. Mitochondria were suspended in the incubation buffer described in Fig. 1 supplemented with 0.3 μM of rhodamine 123 (A only). $\Delta\psi$ and Ca^{2+} fluxes (A and B) were measured after the addition of 6 mM succinate, followed (arrow) by either 20 μM CUR (line b), 10 μM CCCP (line c) or 20 μM CUR+ 1 μM CsA (line d). Line a: No addition of CUR.

3.2. Effect of curcumin on mitochondrial respiration

Fig. 3A shows a polarographic determination of oxygen consumption in mitochondria energized either with the complex I substrate succinate or the complex II substrates pyruvate plus malate. CUR was introduced 90 s after O_2 consumption had reached its steady rate level. CUR increased the basal O_2 consumption when succinate was used as a substrate but only a very small or no effect was observed in the presence of pyruvate plus malate. It should be noted that only a moderate

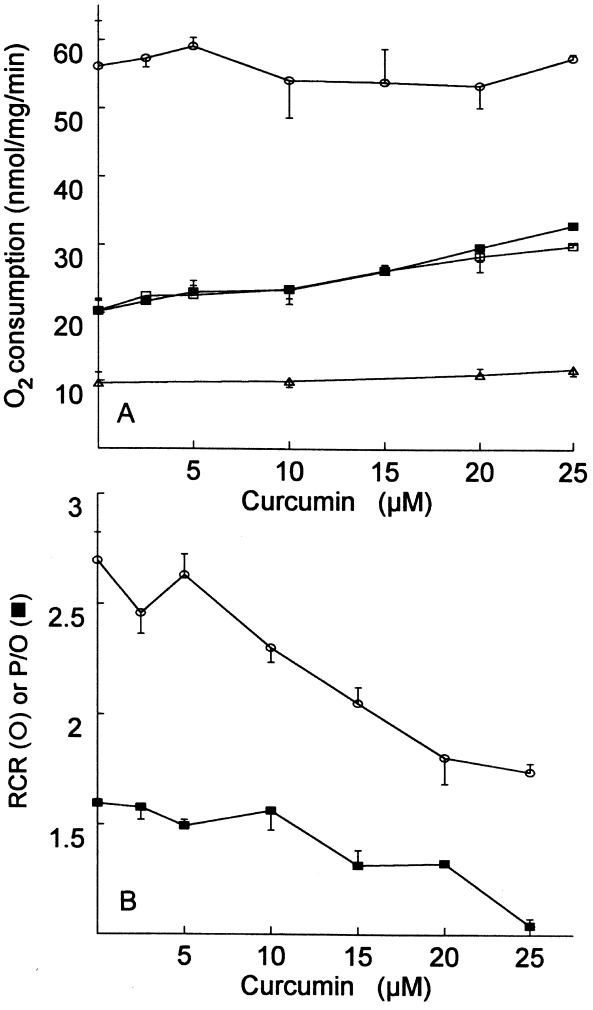

Fig. 3. Alteration of mitochondrial functional parameters in the presence of increasing concentrations of curcumin. Mitochondrial respiration (1 mg of protein/ml) was induced by the addition of succinate (6 mM) or pyruvate/malate (6/6 mM; A only, trace △) and oxidative phosphorylation was initiated by the addition of 0.2 mM ADP. O_2 consumption recordings allowed the calculation of the basal respiration rate (state 2, □), the rate of state 3 (ADP-stimulated) respiration (○), the rate of state 4 (non-ADP-stimulated) respiration (■), of the respiratory control ratio (RCR = state 3/state 4), and the *P/O* ratio (ADP used divided by O_2 consumed in state 3 respiration).

swelling could be detected in the presence of pyruvate plus malate (Fig. 1A).

These data underlined the predominant role of complex II in the CUR-induced PTP opening. However, the CUR effect also depended on complex I activity. Addition of the complex I inhibitor rotenone delayed mitochondrial swelling (Fig. 1A), which was probably due to the presence of NADH in the mitochondrial matrix. Indeed, under these conditions the oxidation of the pyridine nucleotides was inhibited, they remain fully reduced and can contribute to the prevention of PTP opening.

This indicates that complex I activity is not necessary for, but can modulate, the effect of CUR. This is in agreement with recent observations which demonstrated a regulation of the PTP by complex I in skeletal muscle mitochondria [22].

When we studied the effect of this molecule on oxidative phosphorylation, we observed that the respiratory control ratio (RCR) and *P/O* values were decreased (Fig. 3B). This effect was not due to a direct action of the drug on ATP synthase since the rate of state 3 (ADP-stimulated) respiration remained constant (Fig. 3A) but is, as stated before, related to an increase of the basal O_2 consumption. These data reinforced the hypothesis that CUR induced PTP opening.

Interestingly, the respiratory chain inhibitors antimycin A, myxothiazol and KCN and the uncoupler CCCP, prevented the swelling induced by CUR. This indicates that the CUR effect may be independent of respiratory chain function since the first agents block the electron transfer chain and thus O_2 consumption, whereas uncouplers accelerate O_2 consumption [23,24]. This was confirmed by the experiments depicted in Fig. 4A showing that CUR was able to induce swelling under deenergizing conditions. The common property of these compounds is to collapse $\Delta\psi$ which immediately either prevents Ca^{2+} entry or produces a leak of Ca^{2+} outside mitochondrial matrix. As PTP opening is highly dependent on intramito-

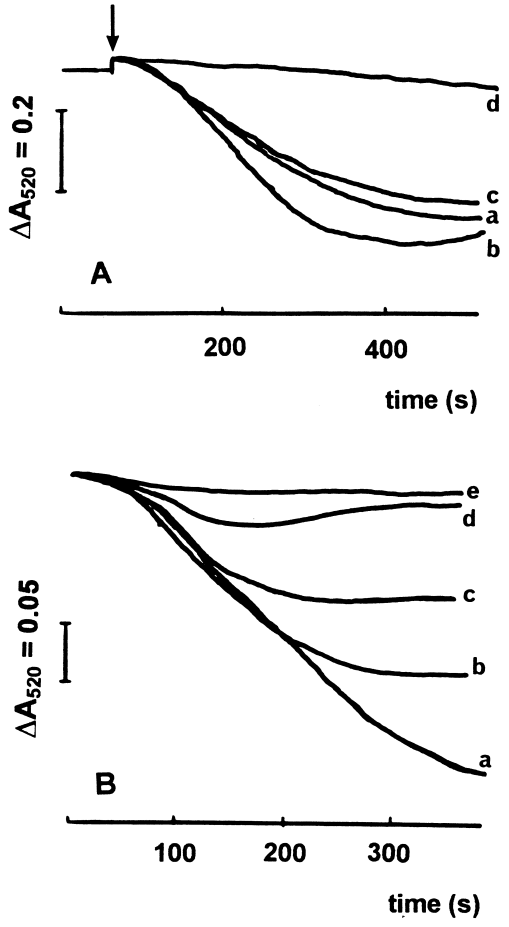

Fig. 4. Dual effect of curcumin (CUR) on mitochondrial swelling in deenergized conditions. Mitochondria were preincubated for 3 min in a buffer containing 0.15 M sucrose, 5 mM Tris–HCl, 2 μM rotenone, 1 μM antimycin A, pH = 7.2 at 25°C. A: Swelling was induced by 20 μM CUR (arrow) in the absence (line a) or in the presence of 20 μM mersalyl (line b), 1 mM dithiothreitol (line c) or 20 μM NEM (line d). B: Swelling was induced by 10 μM t-BH in the presence of 100 μM Ca^{2+} (line a). It was inhibited by increasing concentrations of CUR, 12.5 (line b), 17.5 (line c), 25 (line d) and 50 μM (line e).

chondrial Ca^{2+} concentration [25], we investigated whether the CUR effect was Ca^{2+}-dependent.

3.3. Ca^{2+} plays a crucial role in curcumin effect

When 20 μM EGTA was added to the incubation medium, swelling was completely prevented (Fig. 1B) and $\Delta\psi$ was maintained (Fig. 2A). The same result was obtained when mitochondria were not energized (no Ca^{2+} entry) or when they were preincubated with 1 μM ruthenium red, a well-known inhibitor of the Ca^{2+} uniporter [26], before CUR addition. This shows that the CUR effect required the presence of intramitochondrial Ca^{2+}. It should be noted that only a low Ca^{2+} concentration is required since Ca^{2+} concentration did not exceed 2 μM in our incubation medium.

3.4. Curcumin induces membrane protein thiol group oxidation

Multiple regulatory steps are involved in the modulation of PTP activity. The pore can be opened by a wide variety of agents, especially prooxidant compounds. Lehninger et al. [27] were the first to point out the crucial role of redox phenomenon in the occurrence of PTP. More precisely, oxidation and cross-linkage of membrane protein thiol groups appear to be responsible for membrane permeabilization which was observed with ROS generated by the respiratory chain in the presence of oxidizing or of cross-linker agents [13]. Lipid peroxidation was also hypothesized to participate in the mechanisms leading to pore opening [28]. Thus, we reinvestigated the redox properties of CUR to analyze its effect on PTP. CUR has a complex redox profile. It is an antioxidant agent and a scavenger of free radicals [29] but it has also been suggested that CUR-induced cell death could be mediated by the generation of ROS [8].

Fig. 5A confirmed that CUR inhibited the production of TBARS by liver membranes in a concentration-dependent manner with an IC_{50} value of 0.35 μM. It is interesting to note that for a 20 μM concentration (maximal swelling effect, Fig. 1A), a maximal lipid peroxidation inhibiting effect was observed.

In order to directly assess the potential role of ROS in the induction of pore opening by CUR, we measured their production during mitochondrial swelling. ROS are mainly produced at the level of complexes I and III of the respiratory chain [30]. In normal conditions only about 2–4% of the oxygen consumed is released in the mitochondrial matrix as a superoxide radical ($O_2^{\bullet -}$) which, following dismutation, can generate H_2O_2. In this situation, H_2O_2 may react with mitochondrial Fe^{2+}, resulting in the formation of the hydroxyl radical ($HO^{\bullet}$) [31]. We therefore decided to study the effect of CUR on the production of both $O_2^{\bullet -}$ and H_2O_2. Liver mitochondria energized with succinate produced $O_2^{\bullet -}$. Antimycin A, a complex III inhibitor, stimulated the phenomenon while CUR inhibited $O_2^{\bullet -}$ formation in a concentration-dependent manner (Fig. 5B). It should be noted that CUR did not show any effect on the enzymatic activity of this complex (data not shown) and thus the effect of CUR cannot be related to a direct interaction with the complex III or with the ubiquinone cycle as observed with resveratrol [17]. As we verified that CUR did not reduce nitroblue tetrazolium (the marker used to measure $O_2^{\bullet -}$ production, Fig. 5B) in forma-

Table 1
CUR-induced oxidation of membrane protein thiols

Conditions	thiol groups (%)
Control	100.0 ± 1.8
t-BH 1 mM	75.4 ± 7.3**
CUR 1 μM	94.6 ± 1.2*
CUR 2.5 μM	91.3 ± 1.5**
CUR 5 μM	88.4 ± 2.7**
CUR 10 μM	86.1 ± 5.9**
CUR 15 μM	84.1 ± 5.2**
CUR 20 μM	82.4 ± 3.4**
CUR 30 μM	83.2 ± 6.2**
Succinate 6 mM	99.3 ± 1.3
Succinate 6 mM+CUR 20 μM	87.6 ± 0.9**
Succinate 6 mM+CUR 20 μM+EGTA 100 μM	100.8 ± 2.1
Succinate 6 mM+CUR 20 μM+RR 1 μM	85.9 ± 3.3**

Control value, expressed as 100%, corresponds to 240 nmol of thiol groups/mg proteins. Values represent the means ± S.D. of five experiments in triplicate. *$P < 0.05$; **$P < 0.01$ compared to the control value.
RR, ruthenium red.

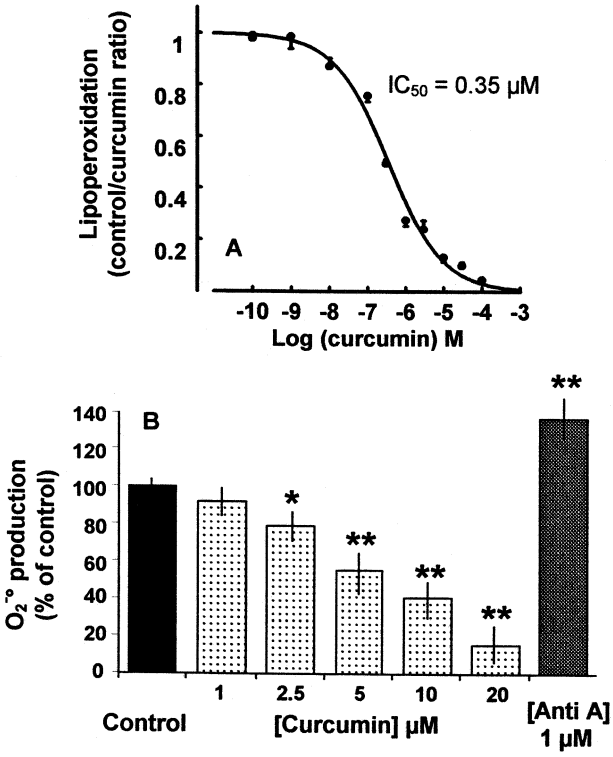

Fig. 5. Inhibition of lipid peroxidation and $O_2^{\bullet -}$ production by curcumin. A: Lipid peroxidation was assessed on liver membranes obtained by differential centrifugation. Liver was homogenized on ice in a Tris buffer (50 mM) using a Teflon Potter homogenizer. The homogenate was centrifuged at 1000×*g* for 10 min and the supernatant was centrifuged for 10 min at 15 000×*g*. The resulting pellet was discarded and the supernatant was centrifuged at 100 000×*g* for 20 min. The final pellet was suspended in NaCl 0.9%. Membranes (0.2 mg/ml proteins) were incubated in a total volume of 1 ml in the presence of a mixture of Fe^{2+}/Fe^{3+} (50 μM/150 μM) for 30 min. The reaction was stopped with 1 ml of 3% trichloroacetic acid and 1.5 ml of 1% thiobarbituric acid was added. The mixture was then heated to 100°C for 30 min and recooled on ice for 10 min before centrifugation in an Eppendorf centrifuge. Then supernatant was read at 530 nm. Results were expressed as μM TBARS/mg of membrane proteins. B: The reaction mixture (25°C) contained mitochondria (0.25 mg protein/ml), 6 mM succinate, 1 μM CsA and 100 μM nitroblue tetrazolium in a final volume of 1 ml of incubation buffer described in Fig. 1. The reaction was started by adding succinate and the rate of nitroblue tetrazolium reduction was measured at 600 nm. Anti A, antimycin A.

zan, the inhibition of $O_2^{\bullet -}$ formation by CUR is probably due to a scavenger effect of CUR towards $O_2^{\bullet -}$. In the same way, CUR greatly diminished H_2O_2 formation, measured using the method of Bagglioni et al. [32], the strongest effect being observed at 20 μM (data not shown). This property was confirmed in experiments on deenergized mitochondria where mitochondrial swelling was induced by a high Ca^{2+} concentration (100 μM) in the presence of *tert*-butylhydroperoxide (t-BH), a generator of alkoxyl or peroxyl radicals. Under these particular conditions, pore opening was prevented by CUR, with complete inhibition observed at 50 μM (Fig. 4B). This clearly demonstrates the duality of the effect of CUR which was able to induce or to prevent pore opening depending on the experimental conditions.

Taken together these experiments confirm that CUR acts as a radical scavenger of ROS and demonstrate that CUR-induced PTP opening is not mediated by an overproduction of these compounds. Therefore, we put the hypothesis of a possible thiol protein oxidation by CUR.

In initial experiments, we investigated the effect of thiol reagents on CUR-induced mitochondrial swelling. As discussed above, thiol oxidation or cross-linking increased PTP opening probability, whereas thiol substitution or disulfide reduction prevented this effect.

The thiol substitution compound *N*-ethylmaleimide (NEM) completely prevented the CUR effect, suggesting that a thiol group is involved in swelling induction. However, in the same experiment monobromobimane, which as NEM forms adducts with glutathione, was inactive. This seems to rule out a possible role of glutathione in the mechanism of action of CUR. This is strengthened by the fact that we were unable to observe any interaction between CUR and glutathione, confirming the data of Oetari et al. [33]. In the same way, the CUR effect was insensitive to the reductant agent dithiothreitol and to the polar monofunctional electrophilic SH reagent, mersalyl (Fig. 4A).

Recently, Costantini et al. [34] made the assumption that PTP opening might be modulated at two separate oxidation sites in equilibrium with both the glutathione and the pyridine nucleotide pools. Therefore, we tested whether the oxidation of pyridine nucleotide was able to influence the CUR effect. The direct determination of pyridine nucleotide oxidation by CUR by classical spectrophotometric and fluorimetric techniques was impossible because both compounds absorb in the same exact spectrum region. However, β-hydroxybutyrate, which has been shown to reestablish pyridine nucleotide levels and to inhibit the swelling induced by the pyridine nucleotide oxidant acetoacetate [34], did not influence CUR-induced swelling. In addition, omission of rotenone accelerated swelling of energized mitochondria, indicating a protective effect of NADH. Thus, the oxidation of pyridine nucleotide did not appear to be the cause of the CUR effect. These data led to the conclusion that oxidation of the glutathione and/or the pyridine nucleotide pools is not a prerequisite to the modulation of PTP opening by CUR. It may act on a site distinct from those described by Costantini et al. [34]. This is in agreement with data suggesting that different PTP inducers can react with different protein thiol groups [35].

In a second series of experiments, deenergized mitochondria were incubated in the presence of increasing concentrations of CUR. Mitochondrial membrane proteins were extracted and the content of the thiol group was determined using Ellman's reagent.

CUR induced a concentration-dependent decrease in free thiol proteins, the maximal effect being observed at 20 μM and equivalent to that obtained with 1 mM of t-BH used as a control (Table 1). Addition of succinate slightly impeded thiol oxidation. This is probably due to the energization of the transhydrogenase which reestablished NADPH to its normal high levels and thus limited oxidation phenomenon [36]. Ca^{2+} is necessary for the CUR effects, since no oxidation could be detected in the presence of EGTA. Interestingly, ruthenium red was unable to prevent the CUR effect. This indicates that intramitochondrial Ca^{2+} was not required to promote thiol group oxidation whereas it was absolutely necessary to induce mitochondrial swelling.

3.5. Mechanism by which curcumin induces PTP

CUR alters mitochondrial function and, more particularly, induces opening of the PTP in liver mitochondria. This is a paradoxical effect for a drug which is a well-known antioxidant described as a cytoprotective agent in different models [37,38]. The present investigation confirms that CUR protects mitochondria against the accumulation of ROS and the lipid peroxidation of membranes and thus in our experimental conditions PTP occurrence cannot be due to the production of ROS which was suggested to induce apoptosis [15]. CUR does not seem to involve an oxidation of either pyridine nucleotides or of glutathione. This is in agreement with the data of Jaruga et al. [39] demonstrating that CUR prevents glutathione loss in dexamethasone-treated thymocytes. The mechanism of action appears to involve a direct interaction with protein thiols of the mitochondrial membrane which are supposed to control PTP opening [31]. The observation that the hydrophobic reagent NEM prevented the CUR effect whereas the more hydrophilic reagents, monobromobimane and mersalyl, were without effect indicates that thiol groups must be embedded in the membrane or localized in its hydrophobic environment. These thiol groups probably belong to cysteine residues [31]. It should be noted that the hydrophobicity of the molecule favors its insertion into biological membranes. The last question is how CUR reacts with thiol groups. Flynn et al. [40] have described the reaction of CUR with several nucleophilic agents (N, S, O) giving birth to additive products. Such a reaction could be considered with two juxtaposed thiols and may represent a possible structure for the thiol–CUR interaction.

Ca^{2+} is necessary to both CUR-induced oxidation and swelling confirming its role in PTP induction. The observation that ruthenium red inhibits mitochondrial swelling without affecting thiol oxidation indicates that intramitochondrial Ca^{2+} was not required to promote thiol groups but was absolutely necessary to induce mitochondrial swelling. This can be due to the binding of Ca^{2+} to sites modulating PTP [25], but also to mobilization of thiol groups by Ca^{2+}, thereby stimulating their reactivity with a prooxidant [35].

In summary, CUR is able to oxidize mitochondrial membrane thiol functions leading to PTP opening in the presence of low Ca^{2+} concentrations. The match between its protective antioxidant effect and its ability to induce PTP, a direct cause of apoptosis, will probably drive the cell to choose between life or death. We can speculate that the issue would depend on

the nature of the cell and could explain the opposite effect of CUR observed with apoptosis [8–11]. This effect may be one of the steps of the mechanism by which CUR induces apoptosis of tumor cells.

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