Cardiolipin mediates curcumin interactions with mitochondrial membranes

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A B S T R A C T

Curcumin, the main molecular ingredient of the turmeric spice, has been reported to exhibit therapeutic properties for varied diseases and pathological conditions. While curcumin appears to trigger multiple signaling pathways, the precise mechanisms accounting for its therapeutic activity have not been deciphered. Here we show that curcumin exhibits significant interactions with cardiolipin (CL), a lipid exclusively residing in the mitochondrial membrane. Specifically, we found that curcumin affected the structures and dynamics of CL-containing biomimetic and biological mitochondrial membranes. Application of several biophysical techniques reveals the CL-promoted association and internalization of curcumin into lipid bilayers. In parallel, curcumin association with CL-containing bilayers increased their fluidity and reduced lipid ordering. These findings suggest that membrane modifications mediated by CL interactions may play a role in the therapeutic functions of curcumin, and that the inner mitochondrial membrane in general might constitute a potential drug target.

1. Introduction

Curcumin is a polyphenolic compound derived from the Indian spice turmeric. Curcumin has been shown to exhibit a broad range of therapeutic effects against neurodegenerative disorders [1], diabetes [2], cancer [3], as well as anti-oxidative [4] and anti-inflammatory activities [5]. Importantly, most diseases and pathological conditions for which curcumin therapy has been applied involve alterations of cellular energy metabolism, underscoring the important role of the mitochondria, the primary organelle responsible for energy generation and metabolism, as a prime cellular target of curcumin. Indeed, curcumin has been shown to modulate mitochondrial processes. For example, curcumin restored mitochondria oxidative functions in a mouse model [6], and contributed to regeneration of mitochondria function in inflamed tissues of obese mice [7]. In tumor cells, curcumin has been shown to affect mitochondria-inducing apoptotic processes [8]. Curcumin affects other mitochondria-associated processes, including reactive oxygen species (ROS) production, modulation of membrane potential, and activities of mitochondria-associated proteins, such as Bax, Bcl-2, Bcl-xl, ATPase, and others [9–11]. The therapeutic effects of curcumin have been mostly studied in the context of its interactions with intracellular proteins, particularly the proteins involved in apoptotic pathways [12,13]. However, membrane interactions of curcumin, which is a highly hydrophobic compound, may also play important roles in its biological activities. Curcumin interactions with phosphatidylcholine-displaying phospholipids, including 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) [14], 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine (DSPC) [15], and egg yolk phosphatidylcholine (EYPC), have been demonstrated [16]. Curcumin was also shown to remodel lipid domains in biomimetic membranes [17], and perturb lipid film/water interphases [18]. While these and other studies examined the interactions of curcumin with different lipids, its specific interactions with mitochondria-like membranes have not been addressed.

Here, we investigated an uncharted aspect of curcumin biomolecular activity – the role of cardiolipin (CL) in affecting curcumin-
membrane interactions. CL is exclusively localized in the mitochondria membrane of eukaryotic cells and it is also abundant in bacterial plasma membranes [19], pointing to fundamental role of this lipid in energy regeneration and respiration processes. CL adopts an hexagonal molecule structure [20], thereby promoting formation of membrane curvatures that are particularly critical in stabilizing the morphology of mitochondrial cristae and assisting assembly of integral proteins [21]. Many proteins localized in the mitochondrial membrane, including adenine nucleotide transporter [22,23], cytochrome c [24], respiratory enzymes [25], and others [26] are found to be tightly bound to CL. Depletion of CL from the mitochondrial membrane significantly alters respiratory activities of mitochondria [27,28]. Therefore, CL could be a potential target for membranotropic pharmacological agents including curcumin designed to modulate metabolic functions. In this work, we utilized biomimetic and biological membrane models in conjunction with advanced biophysical techniques to explore the role of CL in promoting membrane interactions and bilayer internalization of curcumin.

2. Materials and methods

2.1. Materials

(1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione (Curcumin) was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Cardiolipin (Heart, Bovine) sodium salt (CL), 1,2-dimyristoyl-sn-glycerol-3-phospho(-1′-rac-glycerol) sodium salt (DMPG), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycerol-3-phospho(-1′-rac-glycerol) sodium salt (DOPG), 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N-((lissamine rhodamine B sulfonyl)) (N-Rh-PE), N-Tempoyl Palmitamide (N-TEMPO) and 10-doxyl noadecane were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). HCT116 cells were purchased from ATCC and used up to passage 65 (Manassas, VA, USA). 2-amino-2-(hydroxymethyl) propane-1,3-diol (Trizma Base) was purchased from Sigma-Aldrich (Milwaukee, WI, U.S.A.). MitoTracker Orange CM™Ros obtained from ThermoFisher Scientific (Waltham, MA, U.S.A.)

2.2. Preparation of small unilamellar vesicles

Lipid constituents were dissolved in chloroform/ethanol (1:1) and dried together in vacuum up to a constant weight, followed by addition of Tris buffer (pH 7.4) and probe-sonication for 10 min at room temperature, with 20% amplitude and on/off 59-sec cycles. For all methods, the final total concentration of lipids was 1 mM, except for ESR, when the final lipid concentration was 10 mM.

2.3. Preparation of multilamellar dispersions for differential scanning calorimetry

Non-sonicated multilamellar dispersion (1 mM total lipid concentration) for the differential scanning calorimetry (DSC) measurements (see below) was prepared by dissolving dry lipids in Tris buffer (pH 7.4). Glass beads were then added, and the sample was thoroughly shaking until a homogeneous solution was obtained.

2.4. Preparation of giant vesicles

Giant vesicles (GVs) were prepared through a rapid evaporation method [29]. GVs comprising different lipid compositions were dissolved in 1 mL chloroform in a 250 mL round-bottom glass flask. A quantity of 5 mL Tris buffer (10 mM, pH 7.4) was added carefully with a pipette. The organic solvent was subsequently removed in a rotary evaporator under reduced pressure at room temperature. After evaporation for 4–5 min, the resulting vesicle solution exhibited a turbid appearance and was used on the day of preparation. Curcumin was then added to the mixture of giant vesicles at the final concentrations of 0.4–1.6 µM and its fluorescence was visualized by confocal microscopy (see below).

2.5. Cells growth and mitochondria isolation

Human colon cancer cell line HCT116 was cultured in RPMI medium supplemented with 10% FBS at 37°C in 5% CO2 atmosphere. For mitochondria isolation cells were harvested at 70–75% confluence. About 2 g of cell pellet was rinsed with ice-cold PBS (without Ca2+/Mg2+) and re-suspended in a hypotonic solution (100 mM sucrose, 10 mM MOPS, pH 7.2, 1 mM EGTA) to let cells swell for 10 min on ice according to Panov [30]. The suspension was homogenized in a teflon-glass homogenizer by gentle circular strokes following immediate dilution with hypertonic solution (1.25 M sucrose, 10 mM MOPS, pH 7.2, 1 mL per 10 mL cell suspension, to restore the buffer isotonicity. The mix was diluted with three volumes of isolation buffer (75 mM mannitol, 225 mM sucrose, 10 mM MOPS, pH 7.2, 1 mM EGTA, 0.1% fatty acid free BSA). Cellular debris were precipitated at 980 x g, 4°C for 5 min. Supernatant containing mitochondria was further centrifuged at 10,300 x g, 4°C for 20 min. The resulting crude mitochondria pellet was rinsed by centrifugation at the same parameters in 10 mL of MiR06 respiration buffer (110 mM sucrose, 60 mM K-lactobionate, 20 mM HEPES, pH 7.2, 1 mM KH2PO4, 3 mM MgCl2.6H2O, 0.5 mM EGTA, 20 mM taurine, 0.1% fatty acid free BSA) [31]. The final pellet was re-suspended in 100 µL MiR06 by vortexing and stored on ice in refrigerator. The mitochondrial protein was determined by Bradford Protein Assay.

2.6. Preparation of submitochondrial particles (SMPs)

An aliquot of isolated HCT116 cells mitochondria (40 µg/mL) was diluted with the sonication buffer (250 mM sucrose, 10 mM Tris HCl, 1 mM EDTA, pH 7.0) up to 1 mL volume. The mitochondria suspension was probe sonicated for 20 s at 4°C, with 20% amplitude and 5 s on/off cycles to form the inside-out oriented submitochondrial particles (SMP). SMP protein concentration was determined by Bradford Protein Assay and used at the final concentration of 1 µg/mL. The inner mitochondrial membrane inversion in SMPs was verified by cytochrome c oxidase assay (Supplemental Fig. S1).

2.7. Confocal microscopy

Curcumin interaction with HCT116 mitochondria and GVs was assessed by confocal microscopy. HCT116 cells were seeded on collagen pre-coated 35 mm MatTek glass bottom dishes (MatTek Corp, Ashland, MA, U.S.A.) at a density of 300,000 cells. To visualize curcumin interaction with mitochondria, cells were rinsed with a modified Krebs buffer (137 mM NaCl, 5 mM KCl, 1 mM KH2PO4, 20 mM HEPES, pH 7.4, 1 mM MgCl2, 2 mM CaCl2, 10 mM glucose) and stained with 70 nM MitoTracker Orange CM™Ros dye, (Ex/Em wavelengths 554/576 nm) for 30 min at room temperature. After incubation, cells were rinsed and left in the last wash containing 7 µM curcumin (~ 520 nm auto-fluorescence). Imaging was performed on Olympus FluoView FV1000 confocal laser scanning inverted microscope (Olympus, Melville, NY, U.S.A.) in a sequential mode, using 60 × NA 1.42 PLAPON oil objective. GVs were labeled with curcumin, as described above, and placed in a glass-bottom dish. GVs suspensions were imaged on Axiovert 100 M confocal laser scanning microscope (Carl Zeiss, Jena, Germany) using a 63×/1.4 NA Plan Apochromat DIC oil objective. Curcumin fluorescence was excited with a 488 nm argon laser, emitted light was passed through a BP 580–700 nm filter.
2.8. Curcumin fluorescence

Different compositions of small unilamellar vesicle were prepared by the method described above. Samples for fluorescence measurements were prepared by adding set volumes of curcumin (final concentrations \(-0.8 \, \mu M, \ 2.4 \, \mu M, \ 28 \, \mu M\)) to 1 mL Tris buffer (50 mM, pH 7.4) followed by addition of vesicles (1 mM). Fluorescence was measured on a FL920 spectrofluorometer (Edinburgh Instruments, Livingston, UK) using excitation of 470 nm and emission of \(-505 \, nm\).

2.9. Förster resonance energy transfer

Prior to drying, the lipid vesicles were additionally supplemented with N-Rh-PE at a 500:1 mole ratio (phospholipid: N-Rh-PE). Curcumin was then added to the vesicles with N-Rh-PE at different concentrations (0.8–2.4 \, \mu M). Fluorescence emission spectra were acquired in the range of 490–700 nm at the excitation of 470 nm using a FL920 spectrofluorimeter.

2.10. Electron spin resonance (ESR)

Samples for Electron Spin Resonance (ESR) experiments were prepared using the following spin probes: N-Tempoyl Palmitamide (N-TEMPO) and 10-doxyl nonadecane. The spin-probes were added to the vesicle samples in a molar ratio of 500:1 (phospholipid: spin probe). Final vesicle concentration was 10 mM. Samples were placed in a 20 mm length and 1 mm ID glass capillary, and ESR spectra were recorded on an EPR-mini X-band spectrometer (Spin Ltd., Russia) at room temperature. The modulation 20 G, time constant 0.01 and the microwave power level were chosen at subcritical values of 20 mW in order to reach the best signal/noise ratio.

2.11. Differential scanning calorimetry

The differential scanning calorimetry (DSC) experiments were performed on a VP-DSC microcalorimeter (MicroCal, Northampton, MA, U.S.A.). Curcumin at different concentrations was added to the multilamellar dispersions and heating scans were recorded at a rate of 60 °C/h. The Microcal Origin 7.0 software was used for data analysis. Control measurements carried out in the presence of DMSO at same volumes used with curcumin.

3. Results

3.1. Microscopic visualization of curcumin interactions with membranes

In this work, we studied the interaction of curcumin with biological and biomimetic systems mimicking the inner mitochondrial membrane. In particular, the experiments were designed to examine the role of CL in curcumin interactions with and insertion into the bilayer, and concomitant curcumin-induced modulation of bilayer properties.

Fig. 1A depicts confocal fluorescence microscopy images recorded upon incubation of curcumin with HCT116 cells. The fluorescence microscopy experiments were based upon the intrinsic fluorescence of curcumin for monitoring its relative adsorption onto membranes. The moderate 7 \, \mu M dose of curcumin was chosen throughout cellular assessments of curcumin cytotoxicity. Notably, intracellular distribution of curcumin, emitting strong green fluorescence, appears highly correlated with mitochondria staining using MitoTracker Orange dye. This microscopy result indicates that curcumin adsorbs to a significant extent onto mitochondrial membranes upon insertion into living cells.

Fig. 1B examines curcumin binding to biomimetic giant vesicles (GVs) comprising different lipid compositions designed to mimic different cellular membranes. Importantly, Fig. 1B demonstrates preferential interaction of curcumin with vesicle bilayers containing CL. While concentration-dependent accumulation of curcumin occurred in all types of GVs used, i.e. the pure DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), mixed DOPG/DOPC (1,2-dioleoyl-sn-glycero-3-phospho(-1'-rac-glycerol)) and CL/DOPC vesicles, in the latter GVs the fluorescence intensity was significantly enhanced, indicating greater internalization of curcumin within the CL-containing membranes. The GVs employed in the confocal experiments comprised DOPC as the bulk phospholipid as it enabled higher yield and stability of the vesicles.

3.2. Curcumin affinity to cardiolipin

To examine bilayer insertion of curcumin and its dependence upon lipid composition, we monitored the intensity of curcumin fluorescence (excitation peak at 470 nm) upon association with different lipid membranes. Fig. 2A–C demonstrates the degree of curcumin association and insertion into small unilamellar vesicles (SUbs) comprising di-myristoylphosphatidylcholine (DMPC), an abundant phospholipid in cell membranes, and different concentrations of CL. Fig. 2A or di-myristoylphosphatidylglycerol (DMPG) (Fig. 2B) to account the negative charge contribution. Specifically, the vesicles were incubated with 28 \, \mu M curcumin and its fluorescence emission was recorded immediately after curcumin addition. As depicted in Fig. 2A–B (solid black curve, the intrinsic fluorescence of curcumin (at the intensity maximum of 560 nm) in buffer is very low, reflecting the polar environment of the aqueous solution [32]. However, in the presence of CL/DMPC or DMPG/DMPC vesicles, significant fluorescence enhancement occurred, accompanied by a blue shift of the fluorescence peak to 505 nm, indicating insertion of curcumin into the hydrophobic environment of the bilayers [33]. Importantly, the presence of CL in the bilayer gave rise to more pronounced insertion of curcumin, reflected in the progressively higher fluorescence intensity of the peak at 505 nm (Fig. 2A). This finding is consistent with the confocal microscopy data shown in Fig. 1, underscoring the affinity of curcumin to CL. Fig. 2C demonstrates vesicle incorporation of curcumin at lower concentrations (0.8 \, \mu M and 2.4 \, \mu M, respectively), which better reflect the actual therapeutic scenarios and potential systemic concentrations of this polyphenol [34], in DMPC vesicles containing either no DMPG, 2% DMPG (2:98 molar ratio), or CL. Importantly, the CL content of the latter composition corresponds to that of the inner mitochondrial membrane of mammalian cells [35]. The data demonstrate that at a concentration of 2.4 \, \mu M, curcumin appears to incorporate much more significantly within the CL-containing vesicle bilayers compared to the other vesicles tested, giving rise to higher fluorescence intensity (Fig. 2C, black bars).

We further examined association of curcumin with two biological membrane systems (Fig. 2D) – crude membranes of HCT116 cell suspension (Fig. 2D, white bars) and submitochondrial particles (SMPs, Fig. 2D, grey bars) generated from mitochondria isolated from HCT116 colon cancer cells. The two membrane systems were normalized according to the total protein content. SMPs are constructed through sonication and subsequent resealing, thereby exposing the inner mitochondrial membrane and embedded respiratory proteins [36]. A cytochrome c oxidase assay validated the inside-out orientation of the membrane bilayer in the SMPs (Supplemental Fig. S1). The bar diagram in Fig. 2D reveals significantly more pronounced curcumin fluorescence upon interactions with SMPs (grey bars) vs whole cell membranes suspension (white bars). This trend apparent in all curcumin concentrations tested, is consistent with the GVs data in Fig. 2A–C and likely indicates high affinity of curcumin with cardiolipin in SMPs.

3.3. Curcumin-induced Förster resonance energy transfer in lipid bilayers

The preferential association of curcumin within CL-containing bilayers was confirmed in the Förster resonance energy transfer (FRET) experiments, employing vesicles comprising of different lipid compositions (Fig. 3). The vesicles contained the fluorescent dye 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B
sulfonyl) (N-Rh-PE) serving as an acceptor of the fluorescence transferred from curcumin which constituted the fluorescence donor if incorporated within the bilayer. Importantly, the fluorescent residue of N-Rh-PE is localized within the hydrophobic core of the bilayer albeit close to the headgroup interface, and thus can probe the association of curcumin with the bilayer. When no curcumin was added to the N-Rh-PE-containing vesicles, very low fluorescence emission was observed upon excitation at 470 nm, the excitation wavelength of curcumin (Fig. 3, spectrum A). When curcumin (2.4 μM) was incubated with vesicles comprising only DMPC, a fluorescence emission peak at ~590 nm was apparent (Fig. 3, spectrum B), indicating a relatively low uptake of curcumin within the bilayer, which results in small FRET between curcumin and N-Rh-PE. More pronounced FRET was recorded in DMPG/DMPC vesicles (Fig. 3, spectrum C), ascribed to greater association and insertion of curcumin into the vesicle bilayer in the presence of DMPG. Yet, even more efficient FRET was apparent after adding curcumin to CL/DMPC vesicles (Fig. 3, spectrum D), reflected in the lower emission intensity at ~500 nm and higher intensity at ~590 nm. Similar FRET trends were apparent when additional curcumin concentrations were added to the vesicles formed from different lipid compositions (Supplementary Fig. S2). The FRET data shown in Fig. 3 are consistent with the results presented in Figs. 1–2, indicating that CL has a prominent role in mediating membrane insertion of curcumin.

3.4. Curcumin localization within lipid bilayers

In order to assess the localization and depth of curcumin penetration into the bilayer, and the contribution of CL to curcumin localization in the bilayer we carried out ESR experiments. Fig. 4 presents the ESR results obtained through the utilization of two spin probes localized in different areas of the bilayer membrane. The bar diagrams depict the amplitudes of low-field ratios: \( I_{10}/I_{+10} \), where \( I_{+10} \) represents the spin probe signal in the presence of curcumin and \( I_{+10} \) corresponds to the control measurement in the absence of curcumin. Curcumin is a potent free radical scavenger [37], therefore it should quench the ESR signal of the spin probe in its proximity. Fig. 4A outlines the ESR signal intensity ratios recorded for N-Tempoyl Palmitamide (N-TEMPO) in different vesicles, following the addition of different curcumin concentrations (the representative ESR spectra are shown in Supplementary information, Fig. S3). N-TEMPO is localized within the head-group region of the bilayer, and thus reports on molecular events occurring at the membrane interface [38]. Fig. 4A reveals that the addition of curcumin to vesicles comprising pure DMPC and N-TEMPO probe hardly affects the ESR signal compared to the control sample (prior to addition of curcumin), indicating negligible association of curcumin with the DMPC vesicle surface. However, the N-TEMPO signal in the DMPG/DMPC vesicles underwent greater quenching by curcumin, indicating more pronounced adsorption of curcumin onto the bilayer interface. Importantly, Fig. 4A also demonstrates that the most significant decrease in N-TEMPO signal intensity was recorded in CL/DMPC vesicles, consistent with CL-promoting uptake of curcumin into the headgroup area of the vesicles.

To assess whether curcumin was inserted deeper into the acyl chain region of the bilayer, we carried out ESR experiments utilizing vesicles containing the 10-doxyl nonadecane spin probe (Fig. 4B). The doxyl spin probe in this molecule is covalently linked to the carbon in position 10 of the acyl chain, and thus is localized within the hydrophobic interior of the bilayer. Similar to the ESR experiments utilizing N-TEMPO (Fig. 4A), the 10-doxyl nonadecane data in Fig. 4B demonstrate that the
most significant quenching of the ESR signal occurred in the vesicles which contained CL. These results attest that CL promotes curcumin insertion into the hydrophobic core of the bilayer, although to a lesser extent than in the head-group interface.

3.5. Curcumin affects bilayer organization

To further evaluate the impact of membrane association of curcumin on bilayer organization, we carried out the differential scanning calorimetry (DSC) experiments (Fig. 5). The DSC analysis of multilamellar dispersions (MLDs) of DMPC, DMPG/DMPC (2:98) or CL/DMPC (2:98) demonstrates the modulation of bilayer thermodynamic parameters upon addition of curcumin. Specifically, curcumin induced a significant decrease in the gel-liquid phase transition temperature (ΔTm, Fig. 5A) reflecting misalignment of the DMPC acyl tails associated with increased bilayer disorder [39]. Notably, the most negative ΔTm was apparent in CL/DMPC vesicles, consistent with the data shown in Figs. 1–4, indicating CL-promoted bilayer interactions of curcumin. While curcumin-induced changes in Tm occurred in all three lipid compositions examined (Fig. 5A), more substantial modulations of the enthalpy change (ΔH, Fig. 5B) and phase transition temperatures at half-width (ΔT1/2, Fig. 5C) were recorded in the case of CL/DMPC lipid dispersions. In particular, while the enthalpy of the phase transition did not change upon curcumin incubation with pure DMPC or decreased after curcumin addition to DMPG/DMPC, a significant increase in ΔH was recorded upon addition of curcumin to CL/DMPC multilamellar vesicles (Fig. 5B). This finding ascribes to a more pronounced participation of DMPC in the phase transition process, likely due to CL-curcumin interactions which disengage the CL from the DMPC molecules in the bilayer. Similarly, the most pronounced increase in phase transition temperatures at ΔT1/2 occurred in the case of CL/DMPC vesicles following the addition of curcumin (Fig. 5C). This indicates decreased cooperativity in the phase transition processes, due to lower phospholipid order, as a result of perturbations induced by curcumin. These bilayer disruptions result in lowering the Tm values. The impact of curcumin addition resulted in broadening of the thermograph curve (see DSC thermographs, Supplementary Fig. S4).
4. Discussion

Curcumin is among the most-studied polyphenolic compounds exhibiting medicinal potential. However, despite intensive research effort, the therapeutic activity of curcumin towards diverse diseases has yet to be explained. Notably, since altered metabolism is among the hallmarks of curcumin action, analysis of curcumin interactions with the mitochondria, the key metabolic organelle in the cell, is warranted. While many studies have focused on curcumin interactions with signaling proteins including those associated with mitochondria [9,10,40], fewer studies have investigated membrane activity of curcumin. In particular, since curcumin is highly hydrophobic, its interactions with lipid bilayers are expected to significantly modulate membrane properties. Although there have been reports on curcumin interaction with different lipids, to the best of our knowledge there have been no studies aimed at probing curcumin interactions with CL, the lipid molecule exclusively localized in the inner mitochondrial membrane [35].

The data presented in this work demonstrate preferred association of curcumin with CL in lipid bilayers. Utilizing model vesicles that mimic the mitochondrial membrane and biological model addressed by SMPs, we found that in the presence of CL curcumin readily incorporates in the lipid bilayer, and CL affinity was clearly more pronounced compared to bilayers containing DMPC and DMPG. Specifically, Figs. 1–2 demonstrate higher affinity of curcumin to CL compared to PG, likewise curcumin preference in interacting with mitochondrial over other types of membranes. The preferential interaction of curcumin with CL was also observed in FRET experiments (Fig. 3), although the difference in FRET signals between CL and PG was small.

The fluorescence data suggest that the double negative charges upon CL head-group might be an important, although probably not the only, factor which promotes curcumin association with CL. Indeed, lesser bilayer insertion was apparent in the case of glycerol-displaying phospholipids (i.e. DOPG and DMPG), which might seem surprising as both lipids are anionic. Notably, at physiological pH, only one phosphate residue is protonated in CL [41], therefore under such conditions the negative charge of CL is similar to that of PG. Furthermore, curcumin is a weak acid with pKa > 8.0, therefore it is almost neutral under physiological conditions [42,43]. Accordingly, our experimental data suggest that the interactions between curcumin and CL are likely not solely determined by electrostatic attraction, and possibly involve other structural factors. In particular, curcumin affinity to CL may be related to the propensity of CL to adopt non-lamellar hexagonal phase, thereby possibly facilitating more effective association and insertion into the bilayer.

To decipher the precise location of curcumin within the bilayer, ESR assessment was performed using two probes localized in different bilayer regions. The ESR data confirm that CL assists curcumin insertion both into the bilayer core and, particularly, in the headgroup region (Fig. 4). Earlier studies also reported curcumin localization in the proximity of lipid headgroups [18,44]. Importantly, curcumin internalization within the bilayer induced significant modulation of both lipid organization and cooperative properties of the lipids, apparent in the DSC experiments (Fig. 5).

Fig. 6 illustrates a model accounting for CL-induced membrane interactions of curcumin, based on the experimental data presented in Figs. 1–5. According to the proposed mechanism, CL constitutes a

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**Fig. 4.** ESR signal intensities of different radical probes as a function of curcumin concentration. A) N-TEMPO probe. B) 10-doxyl nonadecane probe.

**Fig. 5.** Thermodynamic analysis of the bilayers upon curcumin addition. DSC parameters recorded for the multilamellar vesicle suspensions are indicated. A) $\Delta T_m$, the difference between phase transition temperatures recorded upon addition of curcumin and control vesicles. B) $\Delta H / \Delta H_0$, the enthalpy ratio in which $\Delta H$ corresponds to the enthalpy change after curcumin addition and $\Delta H_0$ is the control vesicle sample prior to curcumin addition. C) $\Delta T(1/2)_i/\Delta T(1/2)_0$, ratio of widths at a half height of the phase transition peak. White bars: 1:50 curcumin:DMPC molar ratio; grey bars: 1:25 molar ratio.
Crucial anchor promoting bilayer interactions and uptake of curcumin. The preferential interaction of curcumin with CL-containing membrane domains and its relatively more pronounced accumulation near the CL headgroup interface may increase membrane fluidity by relieving the tight packing of phospholipids in the bilayer associated with the hexagonal geometry of CL molecules. Such bilayer transformations may affect the activity of bilayer-embedded enzymes partaking in respiration processes, thereby affecting mitochondria functionality. Indeed, CL-mediated interactions of curcumin with mitochondrial membranes could be an underlying factor responsible for the high efficacy of this compound against broad range of metabolic diseases and pathological conditions. This work opens new avenues for understanding and modulating the therapeutic action of curcumin via alterations of energy metabolism.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in online version.

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Authors contribution

R.J., S.K., M.D., Z.O. conceived and designed the experiments. S.B.Z., S.O., S.K., W.J.S., Z.O. performed experiments. S.B.Z., S.O., M.D., L.J.P., D.E.S., Z.O., R.J. analyzed and interpreted the data. All authors contributed to writing the manuscript.

Appendix A. Supplementary data

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