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Studies on curcumin and curcuminoids. XXIII: Effects of curcumin on liposomal lipid peroxidation

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Summary

The potential of curcumin as a photosensitzer, radical scavenger and iron-chelating agent within an artificial membrane (i.e., liposome) is discussed. Curcumin is found to have a dual effect on liposome stability, manifested as peroxidation of phospholipids in the membrane. Curcumin incorporated in the liposomes acts as a chelating agent of ferric and ferrous ions in the membrane.

Introduction

Recent studies on the natural compound curcumin have demonstrated that in solution this substance has a dual effect in oxygen radical reactions (Kunchandy and Rao, 1989, 1990; Tønnesen, 1989a–d; Tønnesen and Greenhill, 1992). Curcumin can act as a scavenger of hydroxyl radicals or catalyse the formation of hydroxyl radicals depending upon the experimental conditions. This dual effect is comparable to the self-sensitization observed in the photochemical degradation of curcumin (Tønnesen et al., 1986). Complex formation between curcumin and iron and reduction of Fe^{3+} to Fe^{2+} in the presence of curcumin is also observed (Tønnesen and Greenhill, 1992). Curcumin proves to be phototoxic in certain bacterial test systems (Tønnesen et al., 1987). The observed photoactivity makes curcumin a potential photosensitizing drug. Curcumin is shown to have an inhibitory effect on the growth of skin tumors in mice (Huang et al., 1989). The observed effect is partly ascribed to the radical scavenging properties of curcumin. Curcumin is also known for its anti-inflammatory activity (Arora et al., 1971; Rao et al., 1982), but the mechanism(s) of action is neither in this case fully evaluated. Cytotoxic lipid peroxidation seems to be important in the inflammatory process and in carcinogenesis (Girotti, 1990; Yu et al., 1991). Curcumin is reported to possess antioxidant activity (Sharma, 1976; Toda et al., 1985, 1988). A better understanding of the mechanism(s) of action of the curcumin molecule can be obtained from studies on the effect of curcumin on lipid peroxidation of membranes. Investigations have shown that liposomes may undergo membrane

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damage in a fashion analogous to that observed for cell membranes (Kinsky, 1972). Lipid peroxidation is detrimental to the membrane structure and function. This can play a role in the toxic as well as therapeutic effects of drugs. Free radical scavengers are able to protect against loss of (liposomal) membrane integrity. The liposome model can therefore be used to investigate the effect of potential drug compounds on membranes in order to evaluate the mechanism(s) of action related to radical reactions and lipid peroxidation. Effect on permeability changes induced under various experimental conditions can be studied. In the present study the liposome model was used to investigate the role of curcumin as a photosensitizer, as a radical scavenger and as an iron-chelating agent in an artificial membrane.

Materials and Methods

Throughout the experiments the samples were protected from light.

Materials

Curcumin was synthesized after the method described by Pabon (1964). $FeSO_4$ (iron(II) sulphate heptahydrate, > 98%), $FeCl_3$ (ferric chloride hexahydrate, 98%), 1,10-phenanthroline monohydrate (> 99%), methylene blue (89%, recrystallized from ethanol before use) and EDTA (disodium salt dihydrate, > 99%) were obtained from Aldrich-Chemie, Germany.

2-Thiobarbituric acid, phosphatidylcholine from soybean (99%) and trichloroacetic acid (100%) were obtained from Sigma, U.S.A. H_2O_2 (hydrogen peroxide 30%) was obtained from Norsk Medisinaldepot, Norway. Cascade Blue hydrazide, trisodium salt, was obtained from Molecular Probes, OR, U.S.A.

Preparation of liposomes

Liposomes were prepared from phosphatidylcholine (soybean). The components were dissolved in chloroform, evaporated in a flask under reduced pressure and finally suspended in 0.9% NaCl. Liposomes containing Cascade Blue were suspended in a solution of Cascade Blue 10^{-5} M in 0.9% NaCl. Liposomes used for the determination of zeta potential were suspended in distilled water. The lipid concentration was 4 mg/ml in all the samples. Equally sized liposomes (105 ± 20 nm, $n = 30$) were obtained by extruding the liposome preparation 10 times at a pressure less than 10 bar through a two-stacked 0.1 μm polycarbonate filter (Extruder, Lipex Membranes, Inc., Canada). The size was measured by photon correlation spectroscopy with a Coulter N4 MD submicron particle analyzer (Coulter Electronics Ltd, U.K.). Immediately before use the liposomes were eluted through a 10 cm Sephadex column (Sephadex G-25M, Pharmacia, Sweden) with 0.9% NaCl.

Stock solutions

Liposomes containing curcumin: after gel filtration the eluate was diluted in 0.9% NaCl to give an absorbance of 0.5 at 425 nm.

Curcumin liposomes with methylene blue: after gel filtration of liposomes containing curcumin the eluate was diluted in a solution of methylene blue (see below) to give an absorbance of 0.5 at 425 nm.

Reference solutions: the blanks were prepared from liposomes without curcumin. After gel filtration the eluate was diluted with NaCl or a solution of methylene blue to the same volume as the corresponding curcumin-containing liposomes.

Methylene blue: a solution of methylene blue in NaCl was prepared to give an absorbance of 0.5 at 620 nm.

NaCl: 0.9% in water; $FeCl_3$: 2.2×10^{-3} M in water; $FeSO_4$: 2.2×10^{-3} M in water; The solutions of iron salts were further diluted in NaCl. EDTA: 2.2×10^{-3} M in NaCl; 2-thiobarbituric acid: 1% in 0.05 M NaOH; trichloroacetic acid: 2.8% in water; 1,10-phenanthroline: 15 mg/50 ml in methanol; and H_2O_2: undiluted (30%).

Irradiation conditions

The radiation source was a Photo-Irradiator consisting of a 900 W xenon arc lamp with an f 3.4 monochromator (Applied Photophysics Ltd, U.K.), operated at 425 nm with a bandwith of 20

TABLE 1

Composition of the samples tested in the study of curcumin as a photosensitizer of lipid peroxidation (samples 1–3) and as a radical quencher (samples 4–12)

Sample no.	Composition of samples					Series A		Series B	
	Liposomes (3 ml)	$FeCl_3$ (0.5 ml)	EDTA (0.5 ml)	NaCl ad 4.5 ml	H_2O_2 (10 μl)	Irradiated ($A_{532\ nm}$)	Non-irradiated ($A_{532\ nm}$)	Irradiated ($A_{532\ nm}$)	Non-irradiated ($A_{532\ nm}$)
1	x	x		x		0.193	0.069	0.341	0.198
2	x			x		0.038	0.037	0.056	0.053
3	x	x	x	x		0.118	0.055	0.380	0.139
4	x	x		x			0.055		0.154
5	x	x		x	x		0.083		0.385
6	x			x			0.044		0.044
7	x			x	x		0.058		0.047
8	x	x	x	x			0.040		0.076
9	x	x	x	x	x		0.075		0.170
10	x	x		x		0.089	0.089	0.262	0.228
11	x			x		0.051	0.050	0.074	0.064
12	x	x	x	x		0.071	0.060	0.157	0.115

The absorbance measured at 532 nm is the absorbance measured according to the thiobarbituric assay for malondialdehyde. The results are the average of at least three parallels. Series A, curcumin liposomes; series B, liposomes without curcumin; samples 10–12 contain liposomes diluted in a solution of methylene blue.

nm or at 620 nm with a bandwith of 40 nm (samples containing methylene blue). A cylindrical cell (5 ml volume) of spectroscopic grade silica was used. The cell was placed in a thermostat at 20 ± 0.1°C and irradiated for 30 min. The samples were continously stirred.

Lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid (TBA) assay for malondialdehyde (Aust, 1985). To 1.0 ml of the samples were added 1.0 ml 2-thiobarbituric acid solution and 1.0 ml trichloroacetic acid solution. The mixtures were heated at 100°C for 20 min, cooled to 25°C and measured spectrophotometrically at 532 nm. The various combinations tested are given in Table 1.

Spectrophotometer and scanning conditions

Interactions between curcumin liposomes and Fe^{2+} or Fe^{3+} were studied by scanning the solutions of curcumin liposomes (diluted in NaCl to absorbance 0.25 at 425 nm) from 200 nm to 600 nm after addition of iron salts in the concentration range 1.1×10^{-3}–2.2×10^{-6} M. Spectra were recorded before and after addition of EDTA. Corresponding samples without curcumin were used as references.

The spectrophotometer was a Shimadzu UV-260 UV-Visible recording spectrophotometer.

Zeta potential measurements

To liposomes prepared in water was added $FeSO_4$ to give a final concentration of ferrous ions of 5. 5×10^{-5} M. The samples were eluted with water through a Sephadex column immediately before determination of the zeta potential. The zeta potential was measured at two independent angles with a Coulter DELSA 440 (doppler electrophoresis light scattering analyzer) (Coulter Electronics, Inc., U.S.A.).

Reduction of ferric ion to ferrous ion

The reduction of ferric ion to ferrous ion was measured by the 1,10-phenanthroline complexation method (Gutteridge, 1985). The various combinations tested are given in Table 2. The samples were measured spectrophotometrically at 510 nm.

Detection of Cascade Blue

Cascade Blue was detected fluorimetrically with a Perkin-Elmer LS 50 luminescence spectrometer, excitation wavelength, 377 nm; emission wavelength, 418 nm.

Results and Discussion

Oxidative degradation of cell membrane lipids in the presence of molecular oxygen, a sensitizing agent and exciting light could play a role in the therapeutic effects of photodynamic action. Curcumin in solution has been shown to act as a photosensitizer of oxygen radicals (Tønnesen et al., 1986, 1987). Curcumin is lipid soluble and will be incorporated in the membrane of liposomes. In the presence of iron the TBA assay indicated an increase in lipid peroxidation upon irradiation in all the samples as shown in Table 1 (series 1–3). For liposomes containing curcumin the catalytic effect was stronger in samples containing only Fe^{3+} than in samples containing Fe^{3+} in combination with EDTA. Curcumin incorporated in the liposome membrane formed a complex with the ferric ion but curcumin had a lower affinity for Fe^{3+} than EDTA (Fig. 1). It has been proposed that iron ligation by moieties in the membranes favors the generation of 'localized' oxygen radicals (Girotti, 1990). Efficient lipid peroxidation will be obtained when the radicals are formed in close proximity to or at an iron-binding site. This will be the case if curcumin incorporated in the membrane acts as a source of radicals upon irradiation. This can possibly explain the observed increase in photodynamic lipid peroxidation of liposomes containing curcumin compared to the reference solution in the presence iron. In samples containing EDTA the photodynamic lipid peroxidation seemed to be independent of the curcumin content.

The TBA assay gives only an indication of the lipid peroxidation taking place. For further evaluation of the reaction mechanisms (type I or II) in the sensitized reaction the TBA assay should be

used in combination with other methods (Girotti, 1990).

No catalytic effect could be oberved in curcumin liposomes without iron. It has been demonstrated previously that curcumin alone is a weak sensitizer in the photooxidation of unsaturated fatty acids (Schieberle et al., 1984). Curcumin is photolabile and decomposes upon radiation at 425 nm. This is reflected in the fading of the liposome preparations during exposure to light at this wavelength.

Curcumin in solution is shown to have a catalytic effect on the formation of hydroxyl radicals generated from H_2O_2 (Tønnesen and Greenhill, 1992). This effect is slightly increased in the presence of Fe^{3+}-EDTA, while an inhibitory effect is observed if curcumin acts as a chelator of Fe^{3+}. In the present study, the TBA assay indicated an increase in lipid peroxidation in all systems containing H_2O_2 (Table 1, series 4–9). For liposomes containing curcumin the effect was slightly greater in the presence of Fe^{3+}-EDTA. The inhibitory effect previously observed in solutions where curcumin acts as a chelator of ferric ions could not be found in the liposome system. The redox potential of iron is strongly influenced by the ligand environment, which will be different in solution and in a membrane. Addition of hydrogen peroxide had a stronger effect on the lipid peroxidation of samples without curcumin than on curcumin-containing liposomes (Table 1, series 4–9). This might be ascribed to the scavenging effect of curcumin on the hydroxyl radicals formed, and emphasize the dual effect of curcumin in radical reactions. The role of H_2O_2 in the in vivo lipid peroxidation, however, has recently been questioned (Buechter, 1988; Schubert and Wilmer, 1991).

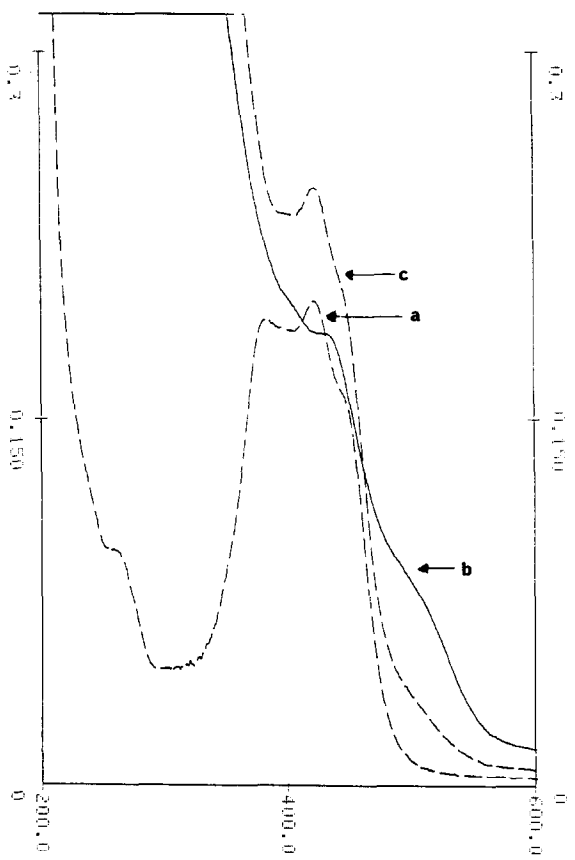

Fig. 1. The absorption spectra of curcumin-containing liposomes (8.2×10^{-6} M curcumin) after addition of ferric ions. (a) Pure liposomes in 0.9% NaCl; (b) liposomes after addition of $FeCl_3$ (2.75×10^{-4} M); (c) liposomes after addition of $FeCl_3$ (2.75×10^{-4} M) and EDTA (2.75×10^{-4} M).

Methylene blue is a water soluble dye which will be present in the adjacent aqueous layer of the liposomes. Methylene blue is a photosensitizer of singlet oxygen and is shown to cause the formation of radical products in liposome systems (Sinclair et al., 1988). Curcumin in solution is a scavenger of singlet oxygen assumed that the samples are protected from light (Tønnesen et al., 1986). This also seemed to be the case when curcumin was incorporated in the liposome membrane (Table 1, series 10–12). An increase in lipid peroxidation could only be observed in samples without curcumin.

Iron may play a role in generating oxidants which can initiate peroxidation. Iron-catalyzed production of oxygen radicals seems to be an important factor in the progression of several diseases, indicating the value of iron chelation treatment (Halliwell and Gutteridge, 1985). The interactions between curcumin-containing liposomes (8.2×10^{-6} M curcumin) and ferric or ferrous ions (2.2×10^{-6}–2.2×10^{-3} M) were studied spectrophotometrically. An interaction between Fe^{3+} or Fe^{2+} and curcumin was ob-

served in all samples by an increase in the absorbance at 500 nm and a decrease in the absorbance at 410 nm (Figs 1 and 2). In the presence of EDTA only a minor change in the absorbance spectra could be detected, demonstrating that EDTA has a higher affinity for both Fe^{3+} and Fe^{2+} than curcumin. This is in agreement with previous results (Tønnesen and Greenhill, 1992). Reduction of ferric ions to ferrous ions was measured by the increase in absorbance at 510 nm (phenanthroline-complex method). The results are given in Table 2. A reduction of Fe^{3+} to Fe^{2+} was observed independent of the curcumin content (Table 2, series 1). After addition of EDTA the reduction of ferric ions to ferrous ions seemed to be most evident in the curcumin-containing samples (Table 2, series 4). This is different from what is observed for curcumin in solution, where no reduction of ferric ions could be detected in the presence of EDTA (Tønnesen and Greenhill, 1992). The redox potential of curcumin is, however, likely to change when the molecule becomes a part of the membrane structure.

Interactions between curcumin-containing liposomes and iron was further reflected in the change in zeta potential when Fe^{2+} was added to the samples. The zeta potential showed a positive increase of 5.9 mV (± 0.2 mV, $n = 4$) in samples containing curcumin liposomes compared to those without curcumin after addition of 5.5×10^{-5} M Fe^{2+}.

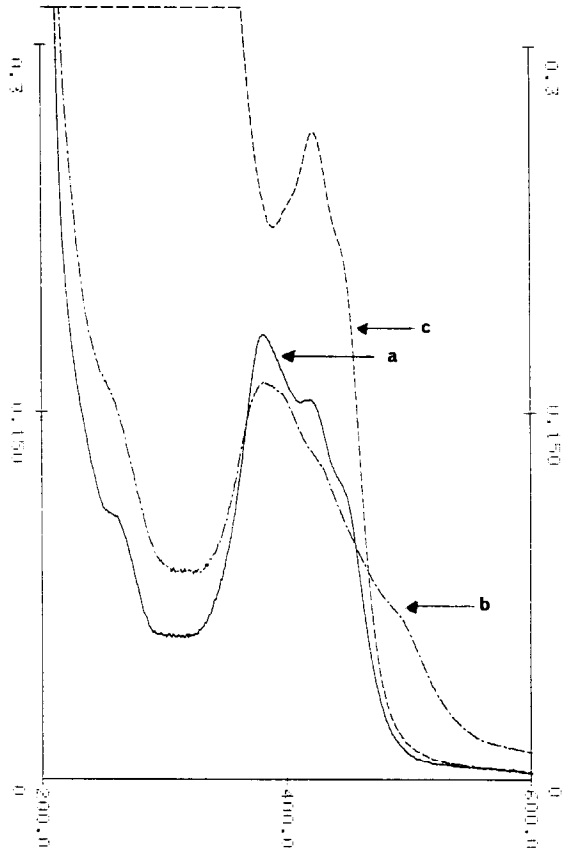

Fig. 2. The absorption spectra of curcumin-containing liposomes (8.2×10^{-6} M curcumin) after addition of ferrous ions. (a) Pure liposomes in 0.9% NaCl; (b) liposomes after addition of $FeSO_4$ (2.75×10^{-4} M); (c) liposomes after addition of $FeSO_4$ (2.75×10^{-4} M) and EDTA (2.75×10^{-4} M).

TABLE 2

Composition of the samples tested in the study of the reduction of Fe^{3+} to Fe^{2+}

Sample no.	Composition of the samples					Series A ($A_{510\,nm}$)	Series B $A_{510\,nm}$)
	Liposomes (5.0 ml)	$FeCl_3$ ($1 \cdot 1 \times 10^{-3}$ M) (2.5 ml)	EDTA (2.5 ml)	NaCl (ad 10 ml)	1,10-Phenanthroline (50 μl)		
1	x	x		x	x	0.108	0.093
2	x			x	x	0.010	0.005
3		x		x	x	0.023	0.024
4	x	x	x	x	x	0.073	0.015
5	x		x	x	x	0.008	0.005
6		x	x	x	x	0.008	0.010

The absorbance at 510 nm is the absorbance measured according to the phenanthroline complex method. The results are the average of at least three parallels. Series A, curcumin liposomes; series B, liposomes without curcumin.

Incorporation of curcumin into the liposome membrane changed the membrane permeability. A fluorescent marker (Cascade Blue) was trapped within the aqueous regions of the liposomes. A fluorescence method was used to measure leakage from the liposomes under various experimental conditions, i.e. non-irradiated liposomes and liposomes irradiated at 425 nm and 620 nm (containing methylene blue). An increase in permeability was observed in curcumin containing liposomes vs. liposomes without curcumin. The degree of leakage did not change as a funtion of irradiation, and there is apparently no correlation between the photooxidation and membrane damage manifested as increased permeability.

Conclusion

Curcumin seems to have a dual effect on the stability of an artificial membrane (i.e., liposome). In combination with light, curcumin within the liposome membrane acts as a sensitizer of oxygen radicals. The resulting photoperoxidation reaction is catalyzed by Fe^{3+}. The biomedical implications of the photosensitizing potential of curcumin should be further evaluated.

Curcumin incorporated in the liposome membrane acts as a scavenger of radicals formed by other photosensitizers like methylene blue. In the absence of light curcumin posesses both catalytic and inhibitory effects on lipid peroxidation caused by hydrogen peroxide. The radical scavenging properties of curcumin might partly explain the observed biological effects of this compound.

Curcumin within the liposome membrane forms chelates with iron. This observation makes curcumin a candidate in ironchelating therapy. The iron-binding properties of curcumin in biological systems are under investigation.

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