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Studies on curcumin and curcuminoids: XXV. Inhibition of primaquine-induced lysis of human red blood cells by curcumin

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Abstract

The protective effect of curcumin on primaquine (PQ)-induced oxidative damage to red blood cells (RBC) is reported. The protection by curcumin against cell lysis was reversed upon increasing curcumin concentration above 20 μM. In contrast, curcumin added as a liposome preparation remained protective even at higher concentrations. Direct determination of curcumin in RBC indicated that the liposomes retained most of the curcumin, thus allowing its incorporation into RBC at concentrations providing stable protective effects against PQ-induced lysis.

Key words: Keywords: Curcumin; Oxidative stress; Red blood cell; Lysis; Liposome; Primaquine

1. Introduction

A set of intracellular or extracellular conditions can lead to the chemical or metabolic generation of oxygen-derived species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$) or lipid peroxides. The human red blood cell (RBC) is exposed to both extracellular and intracellular sources of free radicals. The cell is also rich in polyunsaturated lipids that can undergo lipid peroxidation by active oxygen species and in iron, the latter being a potent catalyst for free radical reactions. The human RBC is therefore a likely target of free radical damage. The RBC are equipped with antioxidant mechanisms to defend themselves against oxygen-derived species, and RBC do not typically undergo oxidative denaturation (Chiu et al., 1989). However, the defending mechanisms might not always be adequate. Oxidative damage to RBC can be induced by drugs (Stern, 1989) or be due to inherited pathology (Saltman, 1989; Shinar and Rachmilewitz, 1990). Oxidative injury to the red cells induces changes in membrane permeability and will eventually result in lysis. The extent of hemolysis has been shown to be related to the degree of intravascular RBC destruction (Chiu et al., 1989). Oxidative damage of human RBC may be prevented or reduced by the use of suitable antioxidants or iron chelators. However, long-term iron-chelating therapy by deferoxamine and administration of large doses of vitamin E (antioxidant) have been tried out in the treatment of

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thalassemia without any improvement in RBC survival (Hershko, 1989; Shinar and Rachmilewitz, 1990). Deferoxamine is water-soluble and will not penetrate the RBC membrane. The failure of vitamin E to improve RBC survival is ascribed to the inability of this compound to prevent peroxidative damage to membrane components other than lipids (Shinar and Rachmilewitz, 1990).

Recent studies of the natural compound curcumin, isolated from the plant *Curcuma longa* L. (turmeric) (Zingiberaceae), have demonstrated that this compound can act as a reducing agent and as a radical scavenger (Tønnesen, 1989a,b; Kunchandy and Rao, 1989, 1990; Tønnesen and Greenhill, 1992; Sreejayan and Rao, 1993). Curcumin incorporated into an artificial membrane (i.e., liposome membrane) acts as a scavenger of radicals and forms chelates with iron, thereby preventing oxidation of the membrane lipids (Tønnesen et al., 1993). Since curcumin possesses both chelating and antioxidant properties, it should have the capability of reducing oxidative damage in RBC. Toxicological studies of this compound indicate that curcumin is non-toxic even at high concentrations (Ammon and Wahl, 1991). Curcumin is practically insoluble in water at acidic or neutral pH. At pH above the lowest pK_a value (i.e., pH 7.8), a water-soluble but unstable form of the molecule exists (Tønnesen and Karlsen, 1985a,b). To be able to bring curcumin to the RBC at an effective concentration the compound must exhibit some aqueous solubility. 1–2% of DMSO can be used as cosolvent in the incubation medium without influencing RBC lysis. This amount of DMSO is sufficient to bring some curcumin in solution in the incubation medium. However, a water-soluble form of curcumin can also be prepared by incorporation of the compound into liposomes. Although the pH of the incubation medium is kept below the lowest pK_a value of curcumin, hydrolytic degradation of the molecule resulting in the formation of feruloylmethane can be observed when curcumin is administered to the medium both as a solution in DMSO and in a liposome formulation.

This study was undertaken to evaluate the protective effect of curcumin on oxidative damage to human RBC. Feruloylmethane, the main degradation product formed by hydrolysis of curcumin, is also a potential antioxidant and was therefore included in this study. The antimalarial drug primaquine is known to induce lysis of RBC in vitro (Cohen and Hochstein, 1964; Grinberg et al., 1992). Primaquine-induced RBC lysis was used as a model of oxidative stress in the testing of the potential antioxidants.

2. Experimental

2.1. Synthesis of curcumin and feruloylmethane

Curcumin was synthesized according to the method of Pabon (1964). The product was recrystallized from methanol. Feruloylmethane was synthesized as described by Nisbet (1938) and purified by use of preparative TLC as detailed previously (Tønnesen, 1985a)

2.2. Preparation of liposomes

Liposomes were prepared from phosphatidylcholine (soybean) (Sigma Chemical Co.). The components were dissolved in chloroform, evaporated in a flask under reduced pressure and finally suspended in sterilized water. The lipid concentration was 4 mg/ml in all the samples. The calculated concentration of curcumin and feruloylmethane was 3.3×10^{-4} and 3.1×10^{-4} M, respectively. Each batch of liposomes was quantified prior to use (see below). The encapsulation efficiency of the two active substances was $\geq 95\%$. Equally sized liposomes (105 ± 20 nm, $n = 30$) were obtained by extruding the liposome preparation 10 times at a pressure lower than 10 bar through a two-stacked 0.1 μm polycarbonate filter (Extruder, Lipex Membranes, Inc.). The size was measured by photon correlation spectroscopy with a Coulter N4 MD submicron particle analyzer (Coulter Electronics Ltd). The liposome preparations were stored under nitrogen at 4°C for up to 3 days before use. When the liposome preparations were intended for long-term storage the preparations were freeze-dried: freezing temperature, −20°C; drying phase, 12–15 h;

with temperature steadily increasing from −40 to 20°C. The vials were sealed under vacuum. The freeze-dried liposomes were resuspended in PBS (phosphate-buffered saline: NaCl, 75 mM and Na_2HPO_4/NaH_2PO_4, 75 mM; pH 7.40) immediately before use by treatment with ultrasound for 1 min. Although there is a difference in size between freeze-dried liposomes after resuspension and extruded liposomes the uptake of curcumin in RBC was identical from both preparations.

Liposomes without curcumin or feruloylmethane were used as reference samples.

For stability testing in aquous medium at pH 7.4, the liposome preparations containing curcumin or feruloylmethane were diluted 1:10 in PBS and incubated at 37°C for various times.

2.3. Solutions in DMSO

Curcumin (6.5×10^{-3} M) and feruloylmethane (3.1×10^{-3} M) in DMSO were added to the incubation medium to give a final concentration in the range 10^{-4}–10^{-5} M. For stability testing in aqueous medium at pH 7.4, the stock solutions in DMSO were diluted and incubated in PBS. Quantification of the solutions was carried out as described for liposome preparations (see below).

2.4. Preparation of RBC

2 ml human blood was centrifuged at 1500 rpm (Hettich Universal Centrifuge) for 15 min. The cells were washed four times with 5 ml PBS (centrifugation at 200 rpm) and suspended in PBS to a hematocrit (Ht) of about 50%.

2.5. Incubation

The final concentration of RBC in the incubation medium was 2% (v/v) and the concentration of glucose was 5.5 mM. For induction of lysis primaquine was used at a concentration of 2.25 mM. Primaquine diphosphate ($M_w = 455$) was purchased from Sigma Chemical Co.

Curcumin or feruloylmethane were added as liposomes or as solutions in DMSO to give a final concentration in the range 10^{-6}–10^{-4} M. RBC samples incubated with 'pure' liposomes or DMSO were used as reference solutions. PBS was added to give a final volume of 10 ml.

The plastic tubes were stoppered and placed into a water bath (shaking) for up to 2 h at a temperature of 37°C.

The samples were protected from light throughout the experiments.

2.6. Hemolysis measurements

The samples were centrifuged for 10 min at 2000 rpm. The supernatant was mixed (4:1) with Drabkin's solution ($K_3Fe(CN)_6$, 200 mg; KCN, 50 mg; KH_2PO_4, 140 mg; surfactant Triton X-100, 0.5 ml; diluted with distilled water to 1000 ml with a pH between 7.0 and 7.4) in order to convert all types of hemoglobin to methemoglobin. The latter forms the stable pigment cyanmethemoglobin with a maximum UV absorbance at 540 nm. The absorbance of the samples was recorded at 540 nm on a Shimadzu 260 UV-visible recording spectrophotometer, and percent hemolysis was calculated. To convert the measurements of optical density in each sample to percent hemolysis, it was assumed that erythrocytes in water (0.2% v/v) were 100% hemolyzed after addition of 1 μl/ml Triton X-100. A standard curve was prepared by dilution of a 100% hemolyzed sample with 1 μl/ml Triton X-100.

2.7. Preparation of the samples for quantification of curcumin and feruloylmethane

After incubation the samples were centrifuged at 2000 rpm for 10 min. The cells were washed once with 2 ml PBS. Both the RBC (resuspended in 800 μl PBS) and the supernatant were frozen at −20°C overnight. After thawing (at room temperature) the RBC were resuspended in PBS to give a final volume of 10 ml. The supernatant was diluted to a final volume of 10 ml. All the samples were extracted with 10 ml ethyl acetate under gentle tumbling for 15 min followed by centrifugation at 2500 rpm for 10 min. 7 ml of the organic phase were evaporated to dryness and

the residue was dissolved in 3 ml ethanol prior to further analysis.

The reproducibility of the extraction method used was ±7% ($n = 5$) and ±5% ($n = 4$) for the extraction of curcumin and feruloylmethane, respectively. The recovery of curcumin was 94.4% (97.5–91.3%, $n = 5$) and the recovery of feruloylmethane was 105.5% (100.0–108.3%, $n = 4$) when liposomes were used as 'membrane models'.

2.8. Quantification of curcumin

At curcumin concentrations above 8×10^{-6} M the samples could be quantified by direct absorption measurements. At lower concentrations of curcumin a fluorimetric method had to be applied.

Curcumin extracted from the RBC was detected fluorimetrically with a Perkin-Elmer LS 50 luminescence spectrometer (excitation wavelength, 425 nm; emission wavelength, 547 nm). The signal was not influenced by other components in the extract as determined from quantum yield measurements and second derivative spectra compared to data obtained from pure curcumin in ethanol. The linearity of the analytical method was good (REG = 0.999) in the concentration range 1.9×10^{-7}–1.3×10^{-6} M. Reproducibility was ±2% ($n = 8$).

Curcumin extracted from the supernatant was detected spectrophotometrically with a Shimadzu 260 UV-Visible recording spectrophotometer (detection wavelength, 425 nm).

The linearity of the method was good (REG = 0.999) in the concentration range 8.2×10^{-6}–4.9×10^{-5} M. Reproducibility was ±2% ($n = 5$).

Each batch of liposomes containing curcumin was quantified by dilution of the liposome preparation 1:10 in PBS followed by extraction and spectrophotometric detection as described for incubated samples.

2.9. Quantification of feruloylmethane

Feruloylmethane was detected by means of HPLC. A Spectra-Physics SP 8700 liquid chromatograph was used. The injector system was a Shimadzu Sil-6A autoinjector (injection volume, 20 μl). The detector was a Shimadzu SPD-6AV with a detection wavelength of 340 nm. The stationary phase was Spherisorb 5 ODS Chrompack (particle size 5 μm), prepacked in a 250 × 4.6 mm i.d. column with a 20 mm Supelco LC18 guard column. The mobile phase was water/methanol (70:30) and the flow rate was 1.2 ml/min. The integrator was a Shimadzu C-3RA. The linearity of the method was good (REG = 0.999) in the concentration range 1.7×10^{-7}–6.8×10^{-5} M. The reproducibility was ±4% ($n = 4$).

Each batch of liposomes containing feruloylmethane was quantified by dilution of the liposome preparation 1:10 in PBS followed by extraction and quantitation as described for incubated samples.

3. Results and discussion

Our data (Fig. 1) show that approx. 20-times more curcumin was detected in the RBC when curcumin was dissolved in DMSO than in the case when it was incorporated into liposomes. Equilibrium was reached within an incubation time of less than 30 min. The amount incorporated into the RBC was therefore clearly dependent upon the 'solvent' used for curcumin. Both the phospholipids used in the liposome prepara-

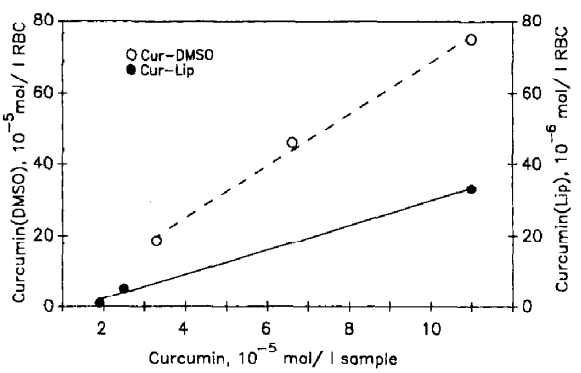

Fig. 1. Accumulation of curcumin in RBC as a function of external curcumin concentration. RBC were incubated with curcumin administered either as a solution in DMSO ((○) REG = 0.98) or incorporated into liposomes ((●) REG = 0.99). The scales of the y-axes for Cur-DMSO and Cur-lip graphs differ by a factor of 10.

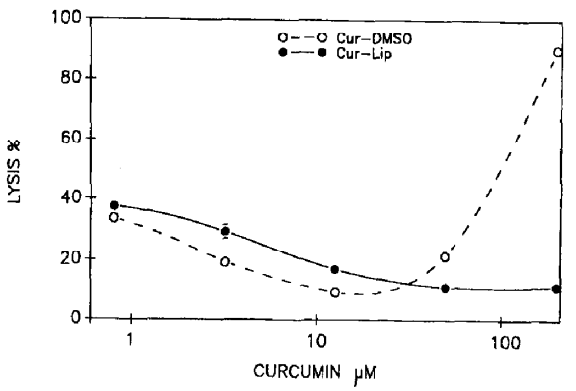

Fig. 2. Dependence of primaquine-induced RBC lysis on curcumin concentration. RBC (2% in PBS) were incubated with PQ (2.25×10^{-3} M) for 2 h at 37°C in the dark. Rate of cell lysis was determined from the ratio of hemoglobin (Hb) released from the cells to the total Hb in the sample. Curcumin was present at the indicated concentrations. (○) Curcumin in DMSO; (●) curcumin incorporated into liposomes (83 μmol curcumin per mg lipid). No lysis was induced by either DMSO (0.25%) or liposomes alone (up to 2.4 mg/ml lipid).

tion and the DMSO can cause changes in the permeability of the RBC membrane. Curcumin itself is lipophilic and will tend to concentrate in the membrane rather than in the cytosol. Accordingly, the compound may accumulate in the RBC membrane at relatively high concentrations. In the presence of liposomes, a considerable proportion of curcumin will be retained in the phospholipid moiety of the liposomes and only a smaller part will reach the cell membrane.

Curcumin was then tested for its antihemolytic activity in vitro. A significant protective effect of curcumin on PQ-induced RBC lysis was detected in both PBS-DMSO and PBS-liposomes systems (Fig. 2). The curves show that the effect becomes significant at micromolar concentrations of curcumin. The effect of curcumin in DMSO reached its maximum at about 20 μM curcumin but reversed upon further increase in curcumin concentration. In contrast, curcumin prepared in liposomes had no such 'toxic phase' and the protection remained stable even at the higher concentrations (Fig. 2). The protective effect of curcumin on RBC lysis correlates with the distribution of the compound between the RBC and the medium (Fig. 1). The toxic phase may be due to the excessive accumulation of curcumin in RBC suspended in a PBS-DMSO solution. The liposomes will retain the bulk of curcumin, thus preventing its accumulation in the RBC membrane at toxic concentrations.

Curcumin has been demonstrated to have a dual effect on lipid peroxidation and $OH^{\cdot}$ radical formation (Kunchandy and Rao, 1989, 1990; Tønnesen et al., 1993). Curcumin may act as a scavenger of $OH^{\cdot}$ formation depending on the experimental conditions. PQ-induced lysis is mediated by H_2O_2 (Cohen and Hochstein, 1964) and probably by membrane-bound iron which catalyses $OH^{\cdot}$ formation from hydrogen peroxide (Haber-Weiss reaction). It is assumed that curcumin, by chelating the iron, changes its reactivity towards H_2O_2 thus inhibiting the Haber-Weiss reaction and possibly lipid peroxidation. At higher concentrations of curcumin reaction with H_2O_2 might give rise to oxidants capable of damaging the cell membrane (Tønnesen and Greenhill, 1992). The assumption that the effect of curcumin on PQ-induced lysis may be explained by the involvement of hydrogen peroxide is supported by an earlier report by Toda et al. (1988). The authors showed that curcumin inhibited H_2O_2-induced lysis of mouse RBC. The concentration-effect curve in those experiments was very close to that in the present work with maximum inhibition at 17.5 μM curcumin followed by a toxic phase.

Curcumin undergoes hydrolytic degradation at pH 7.4 as shown previously (Tønnesen, 1985b). Curcumin in liposomes appeared to be slightly protected towards hydrolytic cleavage compared to a solution of curcumin in DMSO. The amount of curcumin decomposed after 2 h incubation in PBS was 75 and 94% for a liposome preparation and a preparation in DMSO, respectively. A slight reduction in hydrolytic degradation after long-term incubation was also observed in samples containing RBC compared to the corresponding solutions in PBS without RBC. The main hydrolytic decomposition products from curcumin are feruloylmethane and vanillic acid (Tønnesen, 1985a). Vanillic acid is ionised at pH 7.4 and is not likely to penetrate the RBC membrane. Feru-

loylmethane being neutral can possibly diffuse into the RBC membrane. The incorporation of feruloylmethane in red blood cells were therefore investigated. The amount of feruloylmethane formed from curcumin under the given experimental conditions was a direct function of incubation time and curcumin concentration. In the form of liposomes, 0.1% of the original amount of curcumin could be detected as feruloylmethane. In solutions of curcumin in DMSO approx. 30% of the initial curcumin content was detected as feruloylmethane. Feruloylmethane could be detected in all extracts made from RBC incubated with curcumin in DMSO. In these samples the concentration of feruloylmethane was 30–100-times less than that of curcumin in the same cell fraction. Feruloylmethane could also be detected in some samples incubated with curcumin liposomes and was found in a concentration 60-times lower than that of curcumin in the same extracts.

RBC were also incubated with feruloylmethane liposomes or with a solution of feruloylmethane in DMSO. The amount of feruloylmethane in the RBC extracts after incubation with liposomes was below the detection limit (1.7×10^{-7} M). In the presence of DMSO 0.5–0.6% of the original amount (3.1×10^{-4}–6.3×10^{-4} M) of feruloylmethane could be detected in the cell fraction.

A protective effect of feruloylmethane at concentrations similar to that resulting from the hydrolysis of curcumin during the experiments could not be oberved. Feruloylmethane lacks the diketone moiety of the curcumin molecule that has been shown to be involved in the complexation of iron and in the scavenging of oxygen radicals (Tønnesen and Greenhill., 1992).

Interactions between primaquine and curcumin or feruloylmethane were studied by scanning a solution of primaquine in PBS (2×10^{-5} M) from 200 to 600 nm after addition of the two compounds in DMSO or as liposome preparations. The absorption maximum of primaquine was changed by 12 and 28 nm after addition of curcumin in DMSO and in liposomes, respectively. No change in absorption maximum was observed in the presence of feruloylmethane. This is indicative of an interaction between curcumin and primaquine. Such an interaction can lead to the formation of a curcumin-PQ complex resulting in the inactivation of primaquine as a hemolytic agent, but can also result in the production of more oxidants or lead to the formation of a complex that reacts directly with the RBC membrane lipids. The concentration of PQ in the incubation medium was at least 100-times higher than the most protective concentration of curcumin. If inactivation of PQ took place, increasing amounts of curcumin would have resulted in decreasing lysis of the cells. That was not the case as illustrated in Fig. 2. Formation of a curcumin-PQ complex is therefore not likely to have an inhibitory effect on the cell lysis due to deactivation of PQ.

4. Conclusion

Curcumin strongly influences the resistance of the RBC membrane towards oxidative stress. At low concentrations of curcumin within the RBC membrane, curcumin has a protective effect towards primaquine-induced cell lysis. However, a catalytic effect on cell lysis is observed on increase in the amount of curcumin incorporated into the RBC membrane. This dual influence is comparable to the catalytic/inhibitory effect of curcumin on lipid peroxidation observed previously. A liposome preparation of curcumin can be used as a drug delivery system to maintain a non-toxic concentration of curcumin within the cell membrane.

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