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Studies on curcumin and curcuminoids: XXVI. Antioxidant effects of curcumin on the red blood cell membrane

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Abstract

A natural polyphenol curcumin (Cur) was tested for its antioxidant effects on human red blood cell (RBC) and their membranes. Cur, at concentrations of 4–100 μM protected RBC against H_2O_2-induced lysis and lipid peroxidation (LPO). Cur (100 μM) caused significant inhibition of the LPO in normal RBC ghosts supplemented with exogenous iron and in β-thalassemic (Th) ghosts containing endogenous iron deposits. The Cur protective effect on Th ghosts was much more pronounced when ascorbic acid was added in the system (prior to H_2O_2) aiming to induce partial iron reduction. Cur alone did not change the ratio of Fe^{2+}/Fe^{3+} in these ghosts but significantly inhibited the rate of Fe^{2+} reoxidation by H_2O_2. The results indicate the possibility of Fe^{2+} complexing with Cur leading to a lower iron reactivity towards H_2O_2. Therefore Cur as a membrane antioxidant may protect Th RBC against iron-catalyzed oxidative damage.

Keywords: Curcumin; Red blood cells; Membrane; Lipid peroxidation; Iron; β-Thalassemia

1. Introduction

Curcumin (Cur), a natural polyphenolic compound (Fig. 1) isolated from *Curcuma longa* (turmeric) has been extensively studied in recent years (Kunchandy and Rao, 1990; Tønnesen and Greenhill, 1992; Schaich et al., 1994). Cur was found to inhibit lipid peroxidation (LPO) in biological and artificial membranes presumably by scavenging oxygen free radicals and/or by chelating iron (Toda et al., 1988; Pulla Redy and Lokesh, 1992; Tønnesen et al., 1993). In our previous report, we demonstrated that after incubation with red blood cells (RBC) in vitro, Cur accumulated in the cell membranes and protected them against primaquine-induced lysis (Tønnesen et al., 1994). We suggested that by chelating mem-

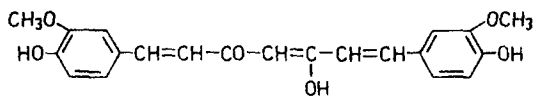

Fig. 1. Chemical structure of curcumin.

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brane-bound iron, Cur reduces its reactivity towards H_2O_2, the most likely causative agent of primaquine-induced lysis. The basis for this assumption came from studies on iron-Cur interactions in chemical and biological systems (Tønnesen and Greenhill, 1992; Schaich et al., 1994; Sreejayan and Rao, 1994).

To test this hypothesis we studied Cur effects on iron-catalyzed LPO in whole RBC and isolated RBC membranes. To elucidate the role of iron in the effects of Cur we used normal ghosts supplemented with exogenous iron and β-thalassemic (Th) RBC ghosts. The latter are known to contain excessive iron deposits (Repka et al., 1993) thus serving as a good experimental model to study iron-catalyzed free radical reactions (Grinberg et al., 1992; Grinberg et al., 1995). Th RBC were shown to have an abnormal lipid composition of their membranes and an increased susceptibility to external oxidants (Stocks et al., 1971; Rachmilewitz et al., 1976).

In this study, we demonstrate that Cur inhibits LPO in whole RBC, in Th RBC membranes and in iron-supplemented normal membranes. We also present evidence that this inhibition may be due to the interaction of Cur with redox active iron in the RBC membrane.

2. Materials and methods

2.1. Reagents

Cur (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione), was obtained from Fluka (Switzerland); 2-thiobarbituric acid (TBA), ferrozine (3-[2-pyridyl]-5,6-bis[4-phenylsulfonic acid] -1,2,4-triazine) and trichloroacetic acid (TCA) were obtained from Sigma Chemical Co. (St. Louis, MO). Exogenous iron was used in a form of complex with nitrilotriacetate (Fe-NTA).

2.2. Blood samples

Blood samples from β-thalassemia patients and healthy controls were collected into EDTA-treated tubes after informed consent. Plasma, platelets and buffy coat were removed by consecutive centrifugations and washings in cold PBS, pH 7.4. In experiments with whole RBC, incubation was carried out in PBS for 2 h at 37°C under continuous shaking in the dark. Each sample contained RBC 2% (v/v), glucose 5.5 mM and PBS 142 mM in a total volume of 2 ml. To induce LPO, the samples were supplemented with H_2O_2 and sodium azide (a catalase inhibitor) at the indicated concentrations. Stock solutions of Cur were prepared by dissolving in DMSO. The final concentrations of Cur in our samples varied from 4 to 100 μM in 1% DMSO. Control samples without Cur contained 1% DMSO. After incubation, the samples were cooled on ice and then LPO was determined according to Rice-Evans et al. (1986).

2.3. RBC ghosts

RBC ghosts were prepared from normal or Th-RBC by subjecting the cells to lysis in cold buffer (5 mM Na-phosphate and 0.5 mM EDTA, pH 8.0) and subsequently five washings with the same buffer (Kuross et al., 1988). Finally, the ghosts were washed twice in 5 mM Na-phosphate buffer, pH 7.4 to remove EDTA. Ghost protein content was assayed by Biorad protein kit (Richmond, USA). The ghosts were then frozen and kept at −70°C until the day of the experiment.

2.4. LPO in RBC ghosts

LPO in RBC ghosts was initiated by H_2O_2 (5.3 mM) in 330 μl of 5 mM Na-phosphate buffer containing 200 μg of ghost protein followed by a 30-min incubation at 37°C in the dark. Normal RBC ghosts were run in two parallel samples with and without Fe-NTA at 20 μM. Th-RBC were preincubated with or without ascorbic acid at 0.5 mM for 60 min prior to addition of H_2O_2. Cur was present in the samples at the indicated concentrations. After incubation, the samples were supplemented with 1 volume of 10% TCA and 1 volume of TBA (0.75% in 0.25 M HCl), boiled for 20 min then cooled and microfuged (Rice-Evans et al., 1986). Absorbance at 532 nm was measured

in supernatants and corrected for TBA-reagent blank (no ghosts). Concentration of malondialdehyde (MDA), a major TBA-reactive product was calculated using $\epsilon_{mM} = 156\ mM^{-1}\ cm^{-1}$ (Buege and Aust, 1978) and expressed per mg of ghost protein.

2.5. Non-heme iron in Th ghosts

Non-heme iron in Th ghosts was determined by its reaction with ferrozine in the presence of sodium dodecyl sulfate (SDS) in 0.2 M acetic buffer as described previously by Kuross and Hebbel, 1988. We measured rapidly-reacting iron by recording absorbance at 562 nm for 2 min after ferrozine addition to the sample in a spectrophotometric cell. In the presence of reducing agents at high concentrations (ascorbic acid at 180 mM and sodium bisulfite at 6 mM), the bulk of iron was in Fe^{2+} form and reacted with ferrozine providing a value of total amount of non-bound iron.

To measure partially reduced iron, we incubated Th ghosts (0.5 mg protein) in 0.5 ml of 5 mM Na-phosphate buffer, pH 7.4 in the presence of ascorbic acid at 2.0 mM with or without Cur for 60 min at 37°C. An aliquot of 0.1 ml was taken for Fe^{2+} determination that was modified as follows. To avoid total iron reduction, ascorbate (180 mM) and Na-bisulfite (6 mM) were omitted from the reaction mixture. Instead, acetate buffer 0.2 M was adjusted with 5% acetic acid to the same pH (3.8) as it had in the presence of the above reducing agents. As a result, these measurements reflected the amount of iron reduced by ascorbic acid (2.0 mM) during the incubation. Two blanks were run in each set of experiments: (i) ghost blank, i.e. all reactants except for ferrozine, and (ii) reagent blank, i.e. all reagents plus ferrozine without ghosts. The sum of these two blanks was subtracted from absorbance at 562 nm for the ghost samples and ferrozine-active iron was calculated using $\epsilon_{mM} = 27.9\ mM^{-1}\ cm^{-1}$ (Kuross and Hebbel, 1988).

3. Results

3.1. Effect of Cur on whole RBC

Hydrogen peroxide (10 mM) incubated with normal RBC in the presence of a catalase inhibitor, sodium azide (1.0 mM) caused considerable lipid peroxidation (Fig. 2). The results show that Cur protected RBC against H_2O_2-induced damage in a dose-dependent manner. In another

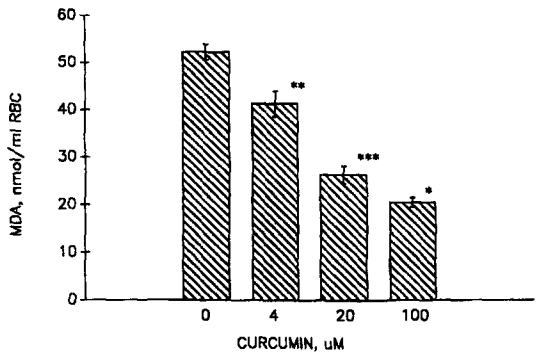

Fig. 2. Effect of curcumin on lipid peroxidation of RBC. Normal RBC were incubated with H_2O_2 (10 mM) and NaN_3 (1 mM) for 2 h at 37°C with Cur at the indicated concentrations. Lipid peroxidation was quantified by TBA-reactive material (MDA). Means ± SEM from eight samples are represented. Significance of the mean differences between two neighbouring bars: $*P = 0.01$; $**P = 0.003$; $***P = 0.0005$.

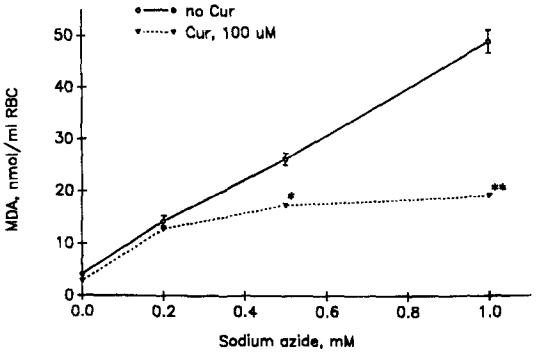

Fig. 3. Effect of curcumin on lipid peroxidation under increasing peroxidative load on RBC. Normal RBC were incubated with H_2O_2 (10 mM) and NaN_3 at the indicated concentrations with or without Cur at 100 μM. Levels of MDA are shown as means ± SEM of five samples. Significance of the mean differences between samples with and without Cur: $*P = 0.001$; $**P = 0.0003$. At several points on the curve, SEM bars are smaller than the symbol size.

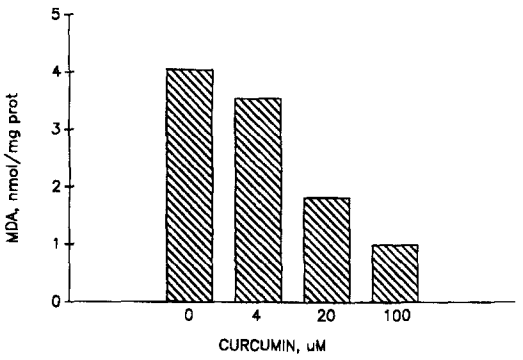

Fig. 4. Effect of curcumin on iron-stimulated lipid peroxidation in normal RBC ghosts. Ghosts (200 μg protein) alone or supplemented with Fe-NTA (20 μM) were incubated with H_2O_2 (5.3 mM) for 30 min at 37°C with Cur at the indicated concentrations. The bars represent differences in MDA levels between samples with and without Fe-NTA. Results of a typical experiment are shown.

series of experiments, RBC were incubated with H_2O_2 at 10 mM and NaN_3 at increasing concentrations with or without Cur (Fig. 3). With higher concentrations of NaN_3 that increasingly inhibited RBC catalase, H_2O_2-induced LPO in RBC was more pronounced. Cur (100 μM) inhibited this process thus demonstrating its protective activity against peroxidative load on RBC.

Notably, after RBC incubation and centrifugation, Cur could hardly be detected in the supernatants as assessed by a loss of the yellow color typical for Cur. This observation is consistent with our recent data (Tønnesen et al., 1994) showing that Cur accumulated in RBC membranes presumably due to its high affinity for cell lipids.

3.2. Effect of Cur on iron-supplemented RBC ghosts

Fig. 4 shows the effect of Cur on iron-stimulated LPO in normal RBC ghosts. The data show the differences in LPO between ghost samples with and without Fe-NTA thus reflecting the interaction of Cur with exogenous iron.

3.3. Effect of Cur on Th RBC ghosts

Six preparations of Th RBC ghosts containing non-heme iron at 30.4 ± 6.0 nmol/mg prot (about 6 μM) which were incubated with H_2O_2 alone produced MDA at 3.4 ± 0.6 nmol/mg. Cur at a concentration of 100 μM inhibited this MDA production by only 32% (Fig. 5) suggesting that the membrane-bound iron in these ghosts has a limited possibility to react with Cur. To increase the proportion of redox active iron capable of reacting with Cur, ascorbic acid (0.5 mM) was added to the ghosts 60 min prior to H_2O_2. This resulted in stimulation of LPO (as detected by MDA) in most of the ghost preparations (Fig. 5). In the presence of both ascorbate and H_2O_2, Cur significantly inhibited MDA formation bringing it down to 1.4 ± 0.2 nmol/mg, i.e. lower than the amount of MDA produced without ascorbate. These results indicated that the membrane iron in reduced form was essential for the protective effect of Cur to be manifested.

To further test this assumption, we measured the rate of iron reduction in Th ghosts in the presence of ascorbate with or without Cur. The total amount of ferrozine-active iron in three ghost preparations was 31.2 ± 2.8 nmol/mg protein. No Fe^{2+} was detected in the ghosts before the addition of ascorbate. Cur alone caused no iron reduction. When Th RBC ghosts were incubated with ascorbic acid (2.0 mM) for 60 min at 37°C, about 30% of total ghost iron was converted to Fe^{2+} as detected by ferrozine. Cur

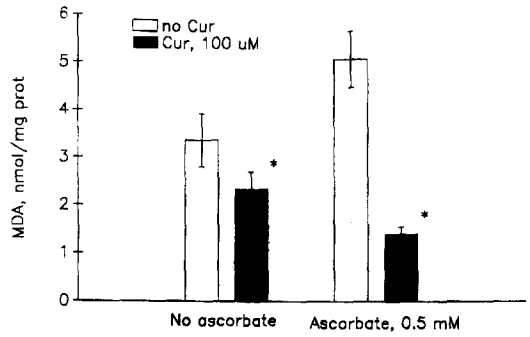

Fig. 5. Protective effect of curcumin against lipid peroxidation in β-thalassemic RBC ghosts. Th-RBC ghosts were preincubated with or without ascorbic acid (0.5 mM) for 1 h at 37°C in the presence or absence of Cur (100 μM) followed by incubation with H_2O_2 (5.3 mM) for another 30 min. MDA levels determined in six ghost preparations are expressed as means ± SEM. *Denotes significant difference (P = 0.03) between samples with and without Cur.

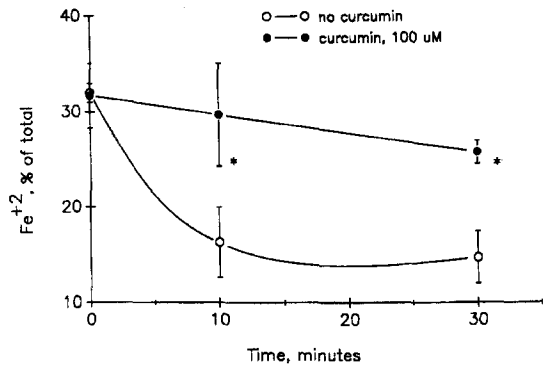

Fig. 6. Effect of curcumin on H_2O_2-induced iron reoxidation in β-thalassemic RBC ghosts. Th-RBC ghosts alone or with Cur at 100 μM were preincubated with ascorbic acid (2.0 mM). The amount of ferrous iron was determined using the ferrozine assay. The samples were then supplemented with H_2O_2 (1.7 mM) to induce iron reoxidation which was monitored by repetitive measurements of Fe^{2+} with ferrozine. Means $\pm$ SEM of three independent experiments are represented. *Denotes significant difference ($P < 0.02$) between samples with and without Cur.

at a concentration of 100 μM did not affect the process of iron reduction, nor did it reoxidize the iron reduced by ascorbate (32 $\pm$ 1% without Cur vs. 32 $\pm$ 3% with Cur). H_2O_2 (1.7 mM) added to these ghosts caused considerable reoxidation of the reduced iron (Fig. 6, lower curve). Cur significantly inhibited this process and partially prevented the reduced iron from reoxidation by H_2O_2 (Fig. 6, upper curve).

We also tested the possibility that Cur, by complexing with iron, removes it from the cell membrane. Whole Th RBC from one patient were incubated with or without Cur (100 μM) for 2 h and then subjected to lysis and ghost preparation. Ferrozine-active iron content was 11.1 nmol/mg in ghosts from non-Cur Th RBC and 11.9 nmol/mg in ghosts prepared from Cur-treated Th RBC, i.e. no removal of iron by Cur was found. It was characteristic for Th RBC incubated with Cur that the membranes remained yellow-colored even after multiple washings in the process of ghost preparation thus indicating considerable Cur binding to Th RBC membranes. The results suggest that the protective effect of Cur against LPO in Th RBC ghosts is not due to iron removal by Cur.

4. Discussion

The bulk of experimental evidence suggests a correlation between RBC susceptibility to external oxidants and their survival in circulation (Stocks et al., 1971; Rachmilewitz et al., 1976; Kuross and Hebbel, 1988; Repka et al., 1993). Therefore, in vitro models of oxidative stress are widely used to study mechanisms of RBC damage and to test potential antioxidants.

Cur was found to inhibit LPO and lysis in mouse RBC challenged with H_2O_2 (Toda et al., 1988). Recently, we demonstrated a protective effect of Cur on primaquine-induced lysis of human RBC (Tønnesen et al., 1994). It was also shown that (i) primaquine-induced RBC lysis is mediated by H_2O_2 and chelatable iron (Grinberg and Samuni, 1994), (ii) Cur is able to complex ionic iron (Tønnesen and Greenhill, 1992) and (iii) the Cur protective effect on LPO may be reversed by increasing exogenous iron (Pulla Redy and Lokesh, 1992). Considering the above, we therefore suggested that the Cur protective effect on the RBC membrane may be attributed to lower reactivity of free iron towards H_2O_2 due to the formation of iron-Cur complex.

In the present work, Cur protected RBC against oxidative damage induced by direct application of H_2O_2. This protective effect was sustained under increasing peroxidation load caused by increasing concentrations of sodium azide, a catalase inhibitor. In line with the commonly accepted mechanism implicating transition metals in LPO catalysis (Saltman, 1989), we assumed that Cur reacts with redox active iron in the cell membrane. Iron of this type is presumably present in catalytical amounts in normal RBC and it reaches abnormally high concentrations in sickle and Th RBC (Repka et al., 1993).

In Th RBC ghosts, Cur significantly inhibited H_2O_2-induced LPO. This effect was much more pronounced in ghosts preincubated with ascorbic acid that produced Fe^{2+} capable of reacting with H_2O_2. In addition, ascorbic acid combined with H_2O_2 might induce iron mobilization thus increasing the pool of redox active iron (Repka and Hebbel, 1991). As a result more iron in its reduced form becomes available to Cur that binds

to Fe^{2+} at the reaction site within these membranes. In the given system, addition of Cur caused no changes in valency either in Fe^{3+} or in Fe^{2+}. This is in agreement with ESR studies (Schaich et al., 1994) showing that the complexation with Cur affected iron redox potential without changing the ratio Fe^{2+}/Fe^{3+}. Our present results imply that Cur inhibits the reaction between H_2O_2 and Fe^{2+}, thus decreasing the rate of LPO in RBC ghosts. This effect may be attributed to Fe^{2+} binding by Cur resulting in lower Fe^{2+} reactivity towards H_2O_2.

The net effect of Cur seems to be determined by its localization in the membrane and by the presence of oxygen and trace metals (Tønnesen et al., 1993; Tønnesen et al., 1994; Schaich et al., 1994). The chemical structure of Cur predicts that the reactions can take place in the aromatic rings of the molecule or in the diketone moiety. Studies on Cur and its analogs suggest that the complex formation with ionic iron involves the diketone moiety and not the phenolic group of the molecule (Tønnesen and Greenhill, 1992; Sreejayan and Rao, 1994). Preliminary data obtained with liposomes indicate that the diketone moiety is more reactive on the surface than inside the lipid phase (not shown). Therefore, it may be suggested that the redox active iron in Th RBC membrane is localized on the membrane surface where the iron-Cur complex formation takes place.

Acknowledgements

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