ABSTRACT

The antioxidant/ pro-oxidant properties of curcumin have been studied by evaluating its ability to protect RBCs from AAPH (2,2'-azobis(2-amidinopropane) hydrochloride) induced oxidative damage. Lipid peroxidation, hemolysis and K+ ion loss in RBCs were assessed respectively by formation of ThioBarbituric Acid Reactive Substances (TBARS), absorbance of haemoglobin at 540 nm and flame photometry. The treatment of RBCs with curcumin showed concentration-dependent decrease in level of TBARS and haemolysis. However in contrast to the above mentioned effects, curcumin in a similar concentration range, did not prevent release of intracellular K+ ions during the process of haemolysis, rather curcumin itself induced its release even in the absence of haemolysis. The ability of curcumin to prevent oxidation of intracellular GSH due to hemolysis showed mixed results. At low concentrations of curcumin (<10 μM) it prevented GSH depletion and at higher concentrations, the GSH levels decreased gradually. These results indicate that curcumin while acting as an antioxidant, also exhibits pro-oxidant activity at high concentrations.

Introduction

Curcumin (diferuloylmethane), a dietary pigment responsible for the yellow colour of turmeric, has been used in traditional medicine. Extensive research within the past one decade has confirmed that curcumin possesses antioxidant activity and mediates anti-inflammatory effects [1,2]. Curcumin has been shown to inhibit lipid peroxidation and effectively scavenge superoxide and peroxyl radicals [3]. It also shows anti-tumour activity, by suppressing the proliferation of a wide variety of tumour cells [4]. In contrast to these studies, some reports suggest that curcumin induces production of Reactive Oxygen Species (ROS) and leads to apoptosis in proliferating cells [5]. Taken together, these contrasting reports suggest that curcumin exhibits both antioxidant and pro-oxidant activities depending on cell type. Therefore in this present work, we have investigated the differential antioxidant/pro-oxidant behaviour of curcumin, by following its ability to protect human RBCs from free radical-induced damages. RBCs are enucleated cells, containing poly-unsaturated fatty acids in their cell membrane [6]. The
Materials and Method

Curcumin, 2,2'-Azobis(2-AmidinoPropane) Hydrochloride (AAPH), and 5,5'-DiThiobis-2-NitroBenzoic acid (DTNB) were of the best purity available and obtained from commercial sources. All the other chemicals were of analytical grade. Blood samples were obtained by venipuncture from healthy volunteers, with strict adherence to the ethical guidelines laid down by the institutional animal ethics committee and RBCs were isolated. Haemolysis of RBCs was initiated by mixing RBCs suspension in Phosphate Saline Buffer (PBS) with AAPH solution (final concentration 50 mM) and incubating the mixture at 37°C for 3 hours. The lipid peroxidation, haemolysis and K+ ion loss in RBCs as a consequence of haemolysis were assessed respectively by formation of Thiobarbituric Acid Reactive Substances (TBARS), absorbance of haemoglobin at 532 nm and flame photometry [7]. The concentration of GSH in RBCs was determined using 5,5'-DiThiobis-2-Nitro Benzoic acid (DTNB) reagent [7]. In order to test the effects of curcumin on the above processes, RBCs were pre-incubated with varying concentrations of curcumin at 37°C for 30 min, washed twice with cold PBS and then subjected to AAPH-induced haemolysis. The IC\textsubscript{50} value i.e. the concentration of curcumin required to inhibit hemolysis or lipid peroxidation by 50%, was determined by plotting the percent haemolysis or TBARS levels respectively, as a function of curcumin concentration and from the plot, the concentration of curcumin required to reduce the activity by 50% was identified. Each experiment was done in triplicate and results are presented as means ± SEM, n = 3.

Results

(i) Inhibition of AAPH-induced haemolysis and lipid peroxidation in human RBCs by curcumin

In the absence of AAPH, RBCs were stable and the haemolysis was negligible. When aqueous suspension of RBCs was incubated with AAPH, about 53% of haemolysis was observed. Fig. 1 shows variation in % haemolysis in RBCs, pre-incubated with increasing concentrations of curcumin (5-50 \textmu M) for 30 minutes at 37°C and then subjected to AAPH-induced haemolysis. It is evident from Fig. 1, that the percent haemolysis gradually decreased with increasing concentration of curcumin, from which the IC\textsubscript{50} value, was found to be 43 ± 5 \textmu M. Inset of Fig.1 shows variation in TBARS level in RBCs, after being subjected to AAPH-induced damage, in the presence and absence of different concentrations of curcumin. The level of TBARS was significantly increased after incubation of RBCs with AAPH as compared to the control sample. In the presence of curcumin, there was gradual decrease in TBARS formation and this inhibition increased with increasing curcumin concentration from 5 to 40 \textmu M, from which the IC\textsubscript{50} value, was found to be 23.2 ± 2.5 \textmu M. The percent haemolysis in RBCs, incubated with curcumin (5-50 \textmu M), in the absence of AAPH was almost identical to that of control sample, indicating that curcumin itself could not induce haemolysis. The incubation of RBCs with curcumin in the absence of AAPH did not show any significant change in the level of TBARS as compared to control sample.
79% of K⁺ ion leakage was observed. Fig. 2 shows the variation in % K⁺ ion leakage from RBCs pre-incubated with increasing concentrations of curcumin (5-40 μM) for 30 minutes at 37°C and then subjected to AAPH-induced haemolysis. It is evident from Fig. 2 that the percent K⁺ ion leakage is lower in curcumin pretreated samples as compared to control sample, however in each case, the K⁺ ion leakage increased with increasing concentration of curcumin. Inset of Fig. 2 shows variation in percent K⁺ ion loss in RBCs after incubation with different concentrations of curcumin (5 to 100 μM) for 3 hours in the absence of AAPH. From the figure it is clear that the percent K⁺ ion loss is significantly higher in curcumin treated samples, as compared to the control sample. The percent K⁺ ion loss was almost identical; nearly 47% at all the concentrations of curcumin (5 to 100 μM) tested in the present study.

(iii) Effect of curcumin on GSH levels in RBCs after hemolysis

Fig. 3 shows change in GSH levels in RBCs after treatment with AAPH and also in the presence of increasing concentration of curcumin (5 to 40 mM). The normal basal level of GSH in RBCs was found to be 2.74 ± 0.05 nmoles/mg of haemoglobin and after incubation with AAPH, the GSH level reduced to about 1.82 ± 0.03 nmoles/mg of haemoglobin. Addition of curcumin to this reaction system, prevented the reduction in GSH content in a concentration-dependent manner up to 10 mM. However at higher concentrations of curcumin treatment, GSH content reduced in a concentration-dependent manner.

Inset of Fig. 3 shows variation in glutathione level in RBCs, after incubation with different concentrations of curcumin (5 to 40 μM) for 3 hours in the absence of AAPH. From the figure it is clear, that the level of glutathione in curcumin treated RBCs was almost constant up to treatment concentration of 10 μM, however beyond that, a concentration-dependent decrease in level of glutathione was observed.
Discussion

Haemolysis of human RBCs is a very good model for studying free radical-induced oxidative damage to membranes and to evaluate the antioxidant activity of new compounds [6]. Therefore, to evaluate the antioxidant activity of curcumin, lipid peroxidation of the membrane fatty acids, loss of haemoglobin and release of intracellular K⁺ ions have been estimated in human RBCs treated with AAPH. The results obtained from these studies indicate, that curcumin protects RBCs from AAPH-induced lipid peroxidation and thereby prevents haemolysis.

Like in lipid peroxidation and haemolysis assays, curcumin did not show progressive inhibition of K⁺ ion loss and GSH depletion under AAPH-treated condition, thus making us unable to estimate their IC₅₀ values. Curcumin treated samples showed lower percent of K⁺ ion loss as compared to AAPH-treated samples. However among them, the percent K⁺ ion loss was increasing with increasing concentrations of curcumin. Therefore, to know whether curcumin itself has any effect on K⁺ ion loss, we looked at the loss of K⁺ ion only in curcumin treated samples. The results clearly suggest, that curcumin at concentration as low as 5 mM drastically increased the loss of K⁺ ions (47%) from RBCs. With increasing curcumin concentration, the percent of K⁺ ion loss remained almost the same, suggesting the saturation effect of curcumin on K⁺ ion loss. The ability of curcumin to induce the K⁺ ion loss may be because of its effect on Na⁺/K⁺ ion channels present on RBCs membranes. This indicates that curcumin may not be acting as a simple antioxidant, but probably has a pro-oxidant effect.

GSH, a tripeptide containing cysteine, is the most abundant thiol present in mammalian cells [7]. During oxidative stress, the cellular pool of GSH is depleted. Exogenously applied antioxidants protect GSH levels in cells by preventing them from being consumed in reaction with free radicals. Our results indicate that the GSH levels come down significantly in RBCs after AAPH incubation, but treatment with curcumin in the concentration range from 5 to 10 μM prevents decrease in GSH level. However, further increase in concentration of curcumin, showed decrease in GSH level, in a concentration-dependent manner. Some reports suggest, that curcumin reacts with free radicals and generates less reactive phenoxyl radical [8]. Therefore, the observed decrease in reduced GSH level in curcumin (>10 mM) pretreated samples, after exposure to AAPH, could also be due to the excess accumulation of phenoxyl radicals and thereby oxidation of cellular reduced GSH pool to GSSG. Hence, this observation suggests pro-oxidant behaviour of curcumin at higher concentrations of treatment. In conclusion, curcumin shows both antioxidant and pro-oxidant activity in RBCs haemolysis model and at high curcumin concentration, it is the later which predominates over the former.

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References


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