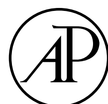

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Curcumin (diferuloylmethane), a singlet oxygen ($^1O_2$) quencher☆

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Abstract

Curcumin (diferuloylmethane) is a major component of food flavoring turmeric (*Curcuma longa*), and has been reported to be anticarcinogenic and anti-inflammatory. Although curcumin was shown to have antioxidant properties, its exact antioxidant nature has not been fully investigated. In this report we have investigated the possible antioxidant properties of curcumin using EPR spectroscopic techniques. Curcumin was found to inhibit the $^1O_2$-dependent 2,2,6,6-tetramethylpiperidine *N*-oxyl (TEMPO) formation in a dose-dependent manner. $^1O_2$ was produced in a photosensitizing system using rose bengal as sensitizer, and was detected as TEMP-$^1O_2$ adducts by electron paramagnetic resonance (EPR) spectroscopic techniques using TEMP as a spin-trap. Curcumin at 2.75 μM caused 50% inhibition of TEMP-$^1O_2$ adduct formation. However, curcumin only marginally inhibited (24% maximum at 80 μM) reduction of ferricytochrome *c* in a xanthine–xanthine oxidase system demonstrating that it is not an effective superoxide radical scavenger. Additionally, there was minor inhibition of DMPO–OH adduct formation by curcumin (solubilized in ethanol) when an ethanol control was included in the EPR spin-trapping study, suggesting that curcumin may not be an effective hydroxyl radical scavenger. Together these data demonstrate that curcumin is able only to effectively quench singlet oxygen at very low concentration in aqueous systems. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Curcumin; Hydroxyl radical; Singlet oxygen; Superoxide anion; Antioxidants; EPR; Spin trapping; TEMP; DMPO

Curcumin (1,7-*bis*(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3,5-dione) also known as diferuloylmethane is a major component of food flavoring turmeric (*Curcuma longa*), and has been reported to be anticarcinogenic and anti-inflammatory [1,2]. Several studies in recent years have shown that curcumin is a potent inhibitor of tumor initiation in vivo [3], and possesses antiproliferative activities against tumor cells in vitro [4]. Furthermore, curcumin has recently been shown to inhibit NFκB activation induced by TNFα [5]. In addition, curcumin also inhibits c-Jun-N-Terminal Kinase (JNK) activation [6]. Curcumin has been shown to inhibit neutrophil activation [7], and suppress mitogen-induced proliferation of blood mononuclear cells [8].

Recent studies have also demonstrated that curcumin is a potent inducer of hemeoxygenase-1 in vascular endothelial cells [9]. Besides these properties, curcumin has also been shown to exhibit antioxidant properties [10,11]. Recent studies have demonstrated that curcumin is a superoxide radical scavenger [10,11], as well as hydroxyl radical scavenger [10,11]. In contrast, curcumin has been shown to photogenerate superoxide anion in toluene and ethanol [12]. Curcumin-induced damage to DNA was prevented by pre-treatment of cells with α-tocopherol, suggesting that curcumin damages DNA through oxygen radical. Moreover, the reaction of curcumin with singlet oxygen, another activated oxygen species has remained controversial. Recent studies also have demonstrated singlet oxygen generation by curcumin in organic solvents [13].

Curcumin was shown to photogenerate singlet oxygen, as well as reduced forms of molecular oxygen under several conditions relevant to cellular environments [14]. Taken together, these studies suggest that curcumin has both antioxidant and prooxidant properties. Although a recent report demonstrated that curcumin could protect

☆ *Abbreviations:* DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; TEMP, 2,2,6,6-tetra-methylpiperidine; $O_2^{-\cdot}$, superoxide anion; OH·, hydroxyl radical; $^1O_2$, singlet oxygen.

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DNA strand breaks caused by singlet oxygen [15]; there has been no direct evidence of singlet oxygen quenching by curcumin in aqueous solutions. Previous studies [12,13] have reported inefficient quenching of singlet oxygen in organic solvents such as acetonitrile. In addition, scavenging of hydroxyl radicals by curcumin has been determined using the deoxyrobose degradation assay [10] or other chemical assays [11]. Therefore, we sought to determine the reactive oxygen species (ROS) scavenging or quenching properties of curcumin by EPR spin-trapping technique. EPR spin trapping is a reliable technique to detect activated species of oxygen in aqueous solutions. In this report we demonstrate that curcumin is an effective singlet oxygen quencher in biologically relevant concentrations in aqueous solutions. However, curcumin was not an effective superoxide or hydroxyl radical scavenger.

Experimental procedures

Materials

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Sigma Chemical Co. (St. Louis, MO). The DMPO was purified by stirring aqueous solutions of DMPO (900 mM) with activated charcoal at 10 mg/ml and filtered through 0.22 μm Millipore filter cartridges, and then centrifuged at 2000*g* for 2 min. The purified DMPO did not give any EPR signal when scanned at 45 or 90 mM concentration. Rose bengal, bovine superoxide dismutase, cytochrome *c* (Type III), mannitol, thiourea, and ferric chloride were obtained from Sigma Chemical 2,2,6,6-tetramethyl piperidine (TEMP) was obtained from Arcos. All other materials were purchased at the highest available purity.

Methods

EPR studies. Singlet oxygen was detected as TEMP-$^1O_2$ adduct (TEMPO) using TEMP as a singlet oxygen trap as described previously [16]. Briefly, photolysis studies were performed at room temperature, in the presence of dissolved air, in quartz capillary tubes. Samples (40 μM rose Bengal + 0.05 M TEMP in 0.2 M boric acid–borax buffer, pH 7.8 with or without curcumin) were irradiated for various time periods at a distance of 30 cm from the lens of a Viewlex VR-25 remote controlled slide projector. Some of the samples were also irradiated inside the EPR cavity for kinetic studies. The generation of singlet oxygen was observed as TEMP-$^1O_2$ adduct on a Bruker D-200 X-Band EPR spectrometer. Scan conditions, unless otherwise indicated were as follows: microwave frequency, 9.8 GHz; power, 10 mW; modulation amplitude 1.0 G; modulation frequency, 100 kHz; time constant, 0.64 s; scan time 200 s; receiver gain 4.0×10^5; center field setting at 3483 G. Curcumin or known singlet oxygen quenchers were tested in this system.

Hydroxyl radicals were generated in a Fenton type reaction, and were detected as DMPO–OH adduct [16]. The reaction mixture contained the following reagents at the final concentration: 31 μM H_2O_2, 33.2 μM ferrous ammonium sulfate, 0.83 mM EDTA, 1.12 mM purified DMPO in 0.2 M boric acid–borax buffer pH 7.8. The reaction was initiated by the addition of ferrous ammonium sulfate. Various levels of curcumin, ethanol, or other scavengers of hydroxyl radicals were tested in the above system. EPR scan conditions were similar to that described for the detection of singlet oxygen.

Superoxide dismutase assay. Superoxide dismutase assay was performed as described by McCord and Fridovich [17]. Briefly, the reaction mixture contained 10^{-9} M xanthine oxidase, 10^{-5} M ferricytochrome *c*, 5×10^{-5} M xanthine in 0.05 M potassium-phosphate buffer, pH 7.8 plus 10^{-4} M EDTA, and the rate of reduction of ferricytochrome *c* was monitored at 550 nm, a linear rate of reduction of ferricytochrome *c* was observed (ΔAbs at 0.025/min) for at least 5 min and superoxide dismutase at 0.1 μg/ml was added to inhibit this rate by 50%. Curcumin was added in some of the reactions as a possible SOD mimic.

Results

Effect of curcumin on superoxide anion

Superoxide anion ($O_2^{-\bullet}$) is known to be produced when xanthine oxidase acts on xanthine in the presence of molecular oxygen. The $O_2^{-\bullet}$ radicals so generated can reduce ferricytochrome *c*. Superoxide dismutase inhibits this reaction by effectively competing with ferricytochrome *c* for the flux of $O_2^{-\bullet}$. This reaction has been used as a convenient assay for superoxide dismutase [17]. When 10^{-9} M xanthine oxidase was added to 10^{-5} M ferricytochrome *c*, 5×10^{-5} M xanthine in 0.05 M potassium phosphate, pH 7.8 plus 10^{-4} M EDTA and was monitored at 550 nm, a linear rate of reduction of ferricytochrome *c* was observed (ΔAbs at 0.025/min) for at least 5 min and superoxide dismutase at 0.1 μg/ml inhibited this rate by 50%, indicating that the reduction of ferricytochrome *c* was dependent on $O_2^{-\bullet}$. We tested the effects of curcumin in this system as a possible superoxide dismutase mimic. Curcumin at 80 μM inhibited the rate of ferricytochrome *c* reduction to a maximum of 24% (Fig. 1). These results indicate that curcumin is not an effective superoxide scavenger.

Effect of curcumin on hydroxyl radical

Hydroxyl radicals were generated in a Fenton type system ($Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+}$). As presented in Fig. 2A a well characterized 1:2:2:1 pattern of DMPO–OH with $A_N = AH_B = 14.92$ G was obtained when 33.2 μM ferrous ammonium sulfate (freshly prepared) was added to 31 μM H_2O_2 in the presence of 1.12 mM DMPO and 0.83 mM EDTA in 0.2 M borate buffer, pH 7.8. The EPR signal of DMPO–OH adducts was stable for several minutes. Addition of $^{\bullet}OH$ scavengers inhibited the signal intensity in a dose-dependent manner. Thiourea (20 mM) inhibited the signal almost completely (data not shown). Addition of 20 mM mannitol also decreased the DMPO–OH adduct formation (data not shown). The effect of curcumin was tested in this system. As demonstrated in Fig. 2, there was minor difference in the percent inhibition of DMPO–OH adduct by curcumin or the solvent ethanol. Curcumin is insoluble in water. In addition, curcumin is soluble to a limited extent (10 mM) in absolute ethanol or DMSO (>0.1 M). Ethanol and DMSO are both

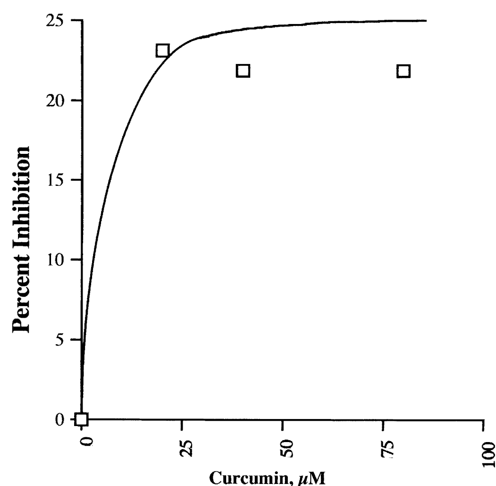


Fig. 1. Effect of curcumin on superoxide radical: superoxide anion was generated in a xanthine–xanthine oxidase system in potassium-phosphate buffer (0.05 M, pH 7.8) as described in Methods. A linear rate of reduction of ferricytochrome *c* was observed for 5 min, which can be inhibited by addition of 0.1 μg superoxide dismutase (SOD). Addition of various concentration of curcumin on the rate of reduction of ferricytochrome *c* was monitored at 550 nm. Inhibition of rate of reduction of ferricytochrome *c* by curcumin was expressed as percent control.

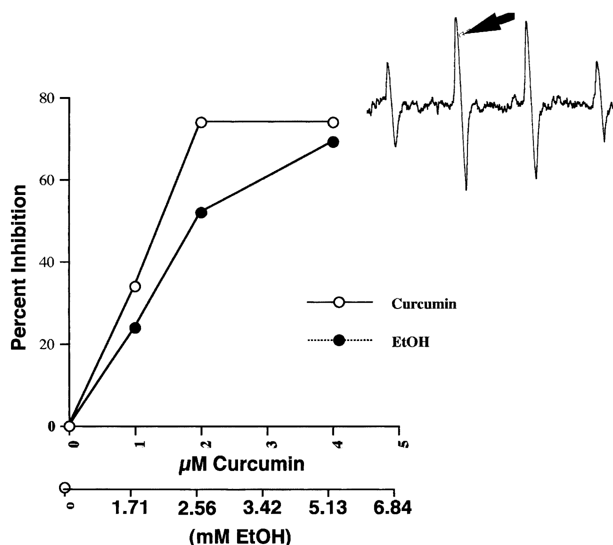


Fig. 2. Effect of curcumin and ethanol on DMPO–OH formation: Hydroxyl radicals were generated and detected as described in Materials and Methods. 31 μM H_2O_2, 1.12 mM DMPO, in 0.2 M borate buffer (pH 7.8) in the presence of 33.2 μM ferrous ammonium sulfate. OH adduct formation was recorded in the EPR spectrometer with the following settings: Receiver gain of 2×10^5, scan rate of 200 s. Other EPR parameters were same as described in "Materials" and "Methods." The percent inhibition was calculated from the intensity of the EPR signal indicated by an arrow. The length of the second peak of the EPR signal was plotted against concentrations of curcumin or ethanol as a percent control.

known to scavenge hydroxyl radicals [18]. In our experiments we have used ethanol as the solvent for preparing a 10 mM stock solution of curcumin, and dilutions were made in absolute ethanol. Therefore, to differentiate the effect of curcumin from that of ethanol, we also included an ethanol control for every concentration of curcumin tested. As demonstrated in Fig. 2, there was no significant difference in the percent inhibition of DMPO–OH adduct formation by ethanol or curcumin dissolved in ethanol. These studies demonstrate that curcumin is not an effective hydroxyl radical scavenger.

Effect of curcumin on singlet oxygen

The generation of $^1O_2$ by photochemical reactions of rose bengal was studied by EPR spectroscopy using TEMP as a spin-trap. We have demonstrated the formation of TEMPO as a nitroxyl radical by the attack of singlet molecular oxygen, generated during photo-activation of various sensitizers, on TEMP [16,18]. The formation of TEMPO as a nitroxyl radical by the attack of singlet molecular oxygen, generated by energy transfer from photo-excited rose bengal on TEMP has been demonstrated previously [18]. The characteristic EPR spectral pattern of three lines of equal intensity for the TEMPO nitroxide radical was observed when air saturated aqueous solution of rose bengal was irradiated in the presence of TEMP at room temperature (Fig. 3A). The hyperfine splitting constants and g-value of the radical were found to be $A_N = 17.2\,G$ and $g = 2.0056$, respectively, consistent with our previously reported values [16,19]. Histidine (10 mM) a well-known singlet oxygen quencher inhibited TEMPO formation (data not shown). We tested the effect of curcumin on TEMPO formation in this system. When various concentrations of curcumin were included in the reaction, and the TEMPO formation was monitored, we observed an inhibition of TEMPO formation in the photo-excitation system. As demonstrated (Figs. 3A and B) addition of curcumin at 3.12 μM in the reaction caused 85% inhibition of TEMPO signal. Curcumin had no effect on TEMPO signal in a control experiment. In addition, curcumin did not inhibit TEMP-$^1O_2$ adduct when added after the samples were irradiated with light. These studies suggest that curcumin inhibits the TEMP-$^1O_2$ adduct by removing $^1O_2$. The concentration of curcumin required to inhibit 50% of TEMPO signal was found to be 2.75 μM (Fig. 3B).

Discussion

In our present report we have shown that curcumin is an effective singlet oxygen quencher as determined by EPR spin-trapping technique. The half maximal inhibition of TEMPO formation by curcumin was 2.75 μM. Curcumin (1–15 μM) did not inhibit superoxide-mediated reduction of ferricytochrome *c* in a xanthine–

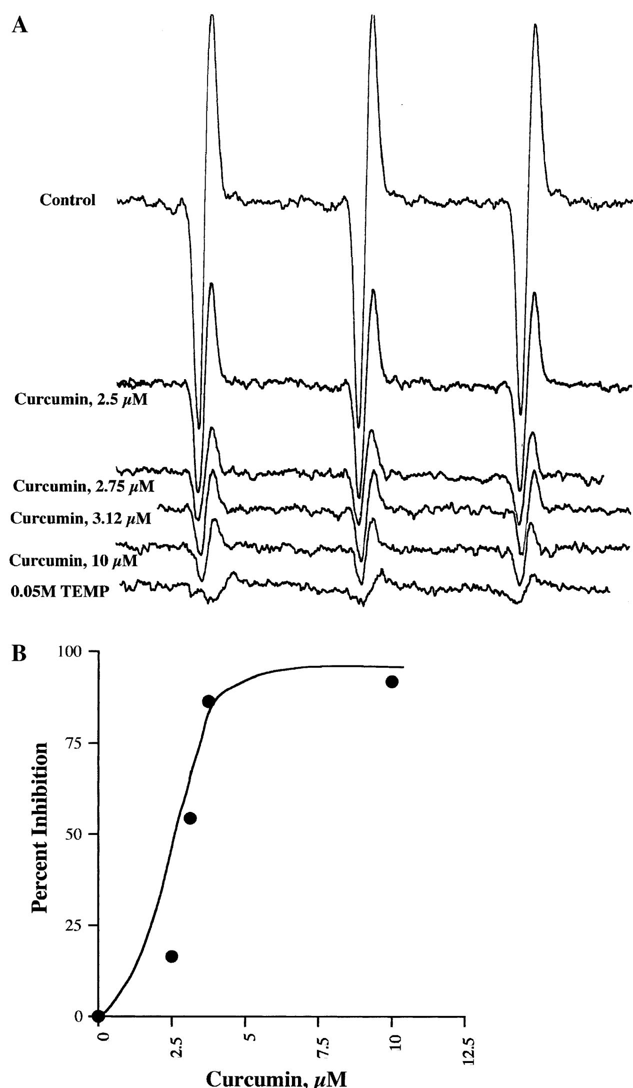


Fig. 3. Effect of curcumin on TEMP-$^1O_2$ adduct formation: (A) effects of curcumin on the formation of TEMP-$^1O_2$ adduct. The experimental conditions was as described under "Materials" and "Methods." Curcumin at indicated concentrations were added to 40 μM rose bengal, 50 mM TEMP dissolved in ethanol (final concentration of ethanol was 10%), in 0.05 M potassium-phosphate buffer, pH 7.8 with 10^{-4} M EDTA. TEMP-$^1O_2$ adduct formation was recorded after irradiating the reaction mixture for 5 min. (B) The percent inhibition was calculated from the intensity of EPR signal of the center peak shown in A.

xanthine oxidase system. However, at higher concentrations (80 μM) curcumin was able to inhibit only 23% in the reduction of ferricytochrome *c*. In addition, there was no significant difference in the inhibition of DMPO–OH adduct formation by curcumin or the ethanol control, suggesting that curcumin may not be an effective scavenger of hydroxyl radicals. Thus, our data demonstrate that curcumin is an effective singlet oxygen quencher at a very low concentration.

Curcumin was earlier shown to scavenge superoxide anion [10,11]. However, the scavenging effect of curcumin could only be observed at very high concentrations. A maximal inhibition of 40% in the reduction of ferricytochrome *c* was observed at 80 μM curcumin [10,11]. In another study a 39% inhibition of reduction of nitroblue tetrazolium was observed by 54 μM curcumin [10,11]. In the present study we observed only a 24% decrease in the rate of ferricytochrome *c* reduction by 80 μM curcumin. Taken together, these observations suggest that curcumin can only partially scavenge superoxide anion at very high concentration, which makes it a poor scavenger of superoxide anion. In cell culture experiments curcumin has been shown to be cytotoxic at greater than 25 μM [20]. Since physiological steady-state concentration of 25 or 50 μM curcumin is difficult to achieve without significant cytotoxicity, curcumin may not be a physiological or pharmacological superoxide scavenger.

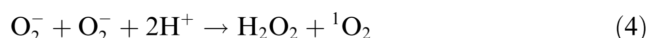
Although previous studies have demonstrated hydroxyl radical scavenging properties of curcumin employing the measurement of TBA reactive substance in a deoxyribose degradation assay [11] or other chemical assays [10], we did not observe effective inhibition of DMPO–OH adduct formation by curcumin. In our system we prepared curcumin in absolute ethanol, and also included same concentration of ethanol in control experiments (Fig. 2). As demonstrated in the Fig. 2 curcumin solubilized in ethanol could inhibit DMPO–OH formation in a dose dependent manner. However, since ethanol is a potent scavenger of hydroxyl radical [20], we measured the effect of ethanol on DMPO–OH adducts formation in control experiments at relevant concentrations. We did not observe significant difference between the ethanol control or curcumin on the inhibition of DMPO–OH formation. These data demonstrate that curcumin is not an effective hydroxyl radical scavenger.

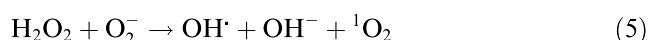
Curcumin inhibited TEMPO formation in a dose-dependent manner (Fig. 3). The amount of curcumin needed to cause 50% inhibition of the rate of quenching $^1O_2$ under these conditions was found to be 2.75 μM. The inhibition of $^1O_2$-dependent TEMPO formation by curcumin detected in our system can be accounted for by proposing the following reaction scheme:

$$\mathrm{Sen} + h\surd \rightarrow \mathrm{Sen}^* \quad (1)$$

$$\mathrm{Sen}^* + O_2 \rightarrow {}^1O_2 + \mathrm{Sen} \quad (2)$$

$$\mathrm{Sen}^* + O_2 \rightarrow O_2^- + \mathrm{Sen} \quad (3)$$


$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + {}^1O_2 \quad (4)$$


$$H_2O_2 + O_2^- \rightarrow OH^{\cdot} + OH^- + {}^1O_2 \quad (5)$$

$$\mathrm{TEMP} + {}^1O_2 \rightarrow \mathrm{TEMPO} \quad (6)$$

As shown in the above scheme, the photosensitizers can be excited (Eq. (1)) which in turn could generate $^1O_2$ by energy transfer pathway (Eq. (2)) or via a minor charge transfer pathway ([14,15]; Eqs. (3)–(5)). The production of singlet oxygen from the spontaneous dismutation of $O_2^{-\cdot}$ (Eq. (4)) in the presence of proton donors has been

demonstrated [18]. TEMP reacts with $^1O_2$ to form TEMPO radical, which is detectable by EPR spectroscopic techniques.

Taken together, the present study demonstrates that curcumin is a potent singlet oxygen quencher at physiological or pharmacological concentration. Additionally, singlet oxygen quenching by low concentration of curcumin in aqueous solutions is a physiologically relevant property of this compound, which can explain its effect in protecting skin against UV light. Singlet molecular oxygen is an electronically excited species of oxygen that is known to be produced in mammalian cells under normal and pathophysiological conditions [21,22]. The photodynamic action of some drugs and pigments is also mediated through $^1O_2$. Light-induced diseases including erythropoietic protoporphyria, pellagra, and cataractogenesis have been attributed in part to the toxicity of $^1O_2$ [23,24]. Thus, curcumin may be used in singlet oxygen-mediated diseases as a pharmacologic agent.

Acknowledgments

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