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THIOCTIC ACID AND DIHYDROLIPOIC ACID ARE NOVEL ANTIOXIDANTS WHICH INTERACT WITH REACTIVE OXYGEN SPECIES

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Thioctic acid (TA) and its reduced form dihydrolipoic acid (DHLA) have recently gained some recognition as useful biological antioxidants. In particular, the ability of DHLA to inhibit lipid peroxidation has been reported. In the present study, the effects of TA and DHLA on reactive oxygen species (ROS) generated in the aqueous phase have been investigated. Xanthine plus xanthine oxidase-generated superoxide radicals (O_2^-), detected by electron spin resonance spectroscopy (ESR) using DMPO as a spin trap, were eliminated by DHLA but not by TA. The sulfhydryl content of DHLA, measured using Ellman's reagent decreased subsequent to the incubation with xanthine plus xanthine oxidase confirming the interaction between DHLA and O_2^-. An increase of hydrogen peroxide concentration accompanied the reaction between DHLA and O_2^-, suggesting the reduction of O_2^- by DHLA. Competition of O_2^- with epinephrine allowed us to estimate a second order kinetic constant of the reaction between O_2^- and DHLA, which was found to be a $3.3 \times 10^5\ M^{-1}s^{-1}$. On the other hand, the DMPO signal of hydroxyl radicals (HO·) generated by Fenton's reagent were eliminated by both TA and DHLA. Inhibition of the Fenton reaction by TA was confirmed by a chemiluminescence measurement using luminol as a probe for HO·. There was no electron transfer from Fe^{2+} to TA or from DHLA to Fe^{3+} detected by measuring the Fe^{2+}-phenanthroline complex. DHLA did not potentiate the DMPO signal of HO· indicating no prooxidant activity of DHLA. These results suggest that both TA and DHLA possess antioxidant properties. In particular, DHLA is very effective as shown by its dual capability by eliminating both O_2^- and HO·.

KEY WORDS: Antioxidant, dihydrolipic acid, free radicals, hydroxyl radical, kinetic constant, lipoic acid, oxy-radicals, superoxide radical, thioctic acid.

INTRODUCTION

Electron reduction of molecular oxygen generates reactive oxygen species (ROS) including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO·). ROS have been implicated in a variety of pathophysiological conditions including aging, cancer, diabetes, heart disease, and various neurological diseases.[1] In experimental settings which mimic ROS-induced injury, many different agents that possess antioxidant properties have been found to be protective against such injuries.[2] Thus, investigations leading to the development of biologically effective antioxidants seem to be very important in protecting against many pathophysiological conditions mediated by ROS.

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Thioctic acid (TA) or lipoic acid, an essential cofactor as lipoamide for mitochondrial alpha-keto-dehydrogenase complexes, has been used as a therapeutic agent in a variety of diseases including liver and neurological disorders. Patients diagnosed with liver cirrhosis, diabetes mellitus, atherosclerosis and polyneuritis have been found to contain a decreased level of endogenous thioctic acid.[3] Since some indications of ROS-induced damage such as lipid peroxidation of membranes have been found as characteristic features of these pathological conditions, it is logical to investigate the antioxidant effects of TA. Several lines of evidence have suggested that the reduced form of TA, dihydrolipoic acid (DHLA) exerts some antioxidant activity against lipid peroxidation.[3,4] Bast and Haenen[5] have suggested a possible antioxidant mechanism of DHLA by hypothesizing that it may reduce glutathione which, in turn, plays a role in vitamin E recycling. In our laboratory, it was recently found, after short term supplementation of rats with a diet enriched with TA, that a significantly increased resistance of tissue homogenates (liver, hearts and others) to lipid peroxidation induction occurred.[6]

A possible mechanism which involves direct protection of membranes by TA/DHLA could have importance. However, because of the complex nature of the mechanism of ROS-induced injury, the possible antioxidant effects of TA and DHLA in the aqueous phase should not be overlooked. To our knowledge, the effects of these agents as antioxidants in aqueous systems have not yet been reported. In the present study, the interactions between TA/DHLA and ROS in the aqueous systems were investigated.

MATERIALS AND METHODS

ESR Measurements

ESR spectra were recorded using an IBM ER200D-SRC electron spin resonance spectrometer in a gas permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm thickness obtained from Zeus Industrial Products, Orangeburg, S.C.) at room temperature. Instument settings: modulation frequency, 100 kHz; modulation amplitude, 1.25 G; scan range, 100 G; central field, 3480 G; microwave power, 9.9 mW; time constant, 500 ms; sweep time 200 /s; gain 10×10^5. Hyperfine coupling constants, a^N and a_β^H were measured directly from the field scan using Mn^{2+} as a marker for calibration.

Measurement of Second Order Kinetic Rate Constant

An apparent second order kinetic rate constant of DHLA with O_2^- was determined from the competition between DHLA and epinephrine for O_2^-. The oxidation of epinephrine (0.5 mM), by xanthine (0.1 mM) plus xanthine oxidate (100 μg), was followed by monitoring the increase of adrenochrome product at the absorbance of 480 nm on a Shimadzu UV160U spectrophotometer in 150 mM KH_2PO_4-KOH (pH 7.4). The second order kinetic rate constant of $4.0 \times 10^4\ M^{-1}\ s^{-1}$ for the reaction between O_2^- and epinephrine was used for calculation.[7]

Xanthine Oxidate Acitivy

Activity of xanthine oxidase was determined by monitoring the formation of urate from xanthine followed at 295 nm on a Shimadzu UV160U spectrophotometer,[8] in 150 mM KH_2PO_4-KOH (pH 7.4).

Sulfhydryl Content

Sulfhydryl (– SH) content was determined by measuring the increase in absorbance at 412 nm using a Shimadzu UV160U spectrophotometer after reaction with 200 μM 5,5′-dithiobis(2-nitrobenzoic acid), DTNB,[9] in 150 mM KH_2PO_4-KOH (pH 7.4). The – SH content was calculated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ for the product of the reaction, *p*-nitromercaptobenzoate.[10] Catalase (100 U) was added before the DTNB addition, to prevent oxidation of *p*-nitromercaptobenzoate by H_2O_2, when ROS existed in the media.[11]

Hydrogen Peroxide Determination

H_2O_2 was determined by measuring the change in absorbance at 240 nm using a Shimadzu UV160U spectrophotometer[12] in 150 mM KH_2PO_4-KOH (pH 7.4). A molar extinction coefficient of 39.4 $M^{-1}cm^{-1}$ was used for calculation.

Chemiluminescence Measurements

Chemiluminescence studies employed a Pharmacia-LKB Wallac 1250 luminometer equipped with a BioOrbit Dispenser at room temperature. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma; 2.5 μM) was used as a probe for HO · in a medium containing 150 mM KH_2PO_4 (pH 7.4), 1 μM $FeSO_4$, and 20 μM H_2O_2. Reactions were initiated by the addition of H_2O_2.

Determination of Ferrous Ion

Ferrous ion (Fe^{2+}) was determined by measuring the increase in absorbance at 510 nm using a Shimadzu UV160U spectrophotometer after reaction with 200 μM *o*-phenanthroline[13] in 150 mM KH_2PO_4-KOH (pH 7.4). Fe^{2+} concentration was calculated using a molar extinction coefficient of 11,100 $M^{-1}cm^{-1}$.

Chemicals

Thioctic acid was a kind gift from ASTA Pharma (Frankfurt). Dihydrolipoic acid was from Pharma-Strohein. Xanthine oxidase (EC 1.1.3.22; from cow milk) was obtained from Boehringer Mannheim Biochemicals. Ferrous sulfate was purchased from Mallinckrodt Chemical Works. H_2O_2 was from Fisher Scientific, DMPO was obtained from Diichi Pure Chemicals (Tokyo). Superoxide dismutase (SOD; EC 1.15.1.1, from bovine erythrocytes), catalase (EC 1.11.1.6, from bovine liver), ferric sulfate, DTNB, *o*-phenanthroline, diethylenetriaminepentaacetic acid (DETAPAC) all were from Sigma Chemical Company. The concentration opf DHLA was estimated by measuring its – SH content. TA was dissolved in 150 mM KH_2PO_4-KOH buffer at 45°C and neutralized to pH 7.4 with KOH at 37°C.[14]

Statistical analysis

Means and standard errors were computed. Significant differences between values were computed using Student's t-test at $P < 0.05$.

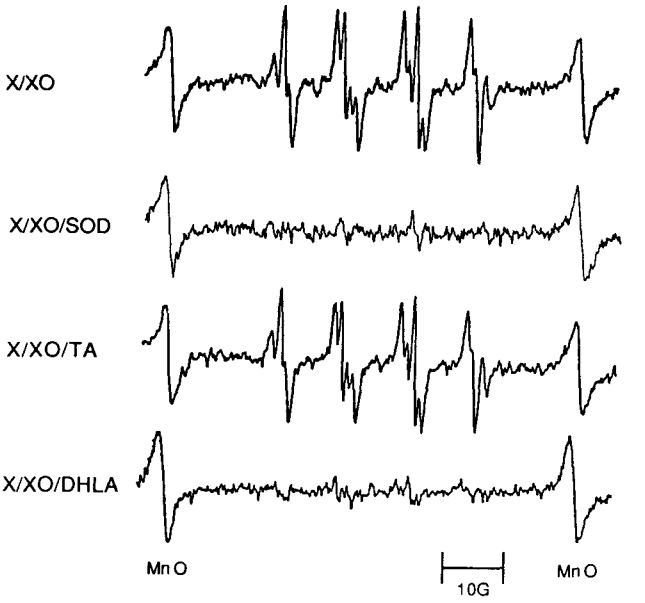

FIGURE 1 ESR study of the effects of thioctic acid and dihydrolipoic acid on superoxide anion radicals generated by xanthine plus xanthine oxidase. DMPO spin adducts generated by 5 mM xanthine (X) plus 100 μg xanthine oxidase (XO) in the presence of 40 μM DETAPAC in 150 mM KH_2PO_4-KOH (pH 7.4) in total volume of 1 ml. [DMPO] = 45 mM; [SOD] = 200 U; [thioctic acid (TA)] = 5 mM; [dihydrolipoic acid (DHLA)] = (5 mM). ESR signals were recorded 3 min after the initiation of xanthine oxidase reaction.

RESULTS

Generation of the DMPO–OOH adduct by 5 mM xanthine plus 100 μg xanthine oxidase in the presence of an iron chelator, DETAPAC (40 μM) is shown in Figure 1. This adduct can be eliminated by SOD, confirming that the adduct indeed originated from O_2^- (second spectrum). The addition of 5 mM TA, before the initiation of the xanthine oxidase reaction, did not have any effects on DMPO–OOH (third spectrum). DHLA at the same concentration, on the other hand, exhibited a total elimination of free radical signals (bottom spectrum). The addition of DHLA after the formation of DMPO–OOH, at the same concentrations of agents, only decreased 10% of the height of the DMPO–OOH signal. DHLA (upto 3 mM) did not have any significant effects on the formation of uric acid by xanthine (50 μM) plus xanthine oxidase (40 μg). These results suggest that DHLA indeed scavenges O_2^-. The apparent second order kinetic rate constant of DHLA with O_2^- was estimated to be $3.3 \times 10^5\ M^{-1}s^{-1}$ from the competition between DHLA and epinephrine for O_2^-. The mechanism of elimination of O_2^- seems to involve the $-SH$ groups of DHLA. As shown in Table I, a significant decrease in –SH content of DHLA was observed after its treatment with 200 μM xanthine plus 40 μg xanthine oxidase for 20 min. Also, a 5-fold increase in the rate of formation of H_2O_2 with 100 μM DHLA was observed by measuring the changes in the absorbance at 240 nm in the presence of xanthine (1 mM) plus xanthine oxidase (40 μg), suggesting the reduction of O_2^- by DHLA.

Figure 2 shows that DMPO–OH adduct cannot be generated with only 2 mM

TABLE I
Effects of superoxide anion radicals on the sulfhydryl content of dihydrolipoic acid

	$-d[SH]/dt$ (nmole/min)
control	0.18 ± 0.03
+ xanthine oxidase + xanthine	0.85 ± 0.32*
+ xanthine oxidase + xanthine + SOD	0.16 ± 0.00

room temperature; pH 7.4; [dihydrolipoic acid] = 100 μM; xanthien oxidase = 40 μg; [xanthine] = 200 μM; [SOD] = 100 U; control contained xanthine oxidase; *significant from control value at $P < 0.05$.

H_2O_2, but when with Fenton's reagent 2 mM H_2O_2 plus 0.2 mM $FeSO_4$ were present, a large signal was obtained (second spectrum). The measurement of coupling constants of the signals generated by $H_2O_2/FeSO_4$ showed that $a^N = a^H_\beta$ suggesting that the signal is indeed DMPO–OH derived from HO·. The third and fourth spectra show that, generation of DMPO–OH adduct by Fenton reaction was inhibited either

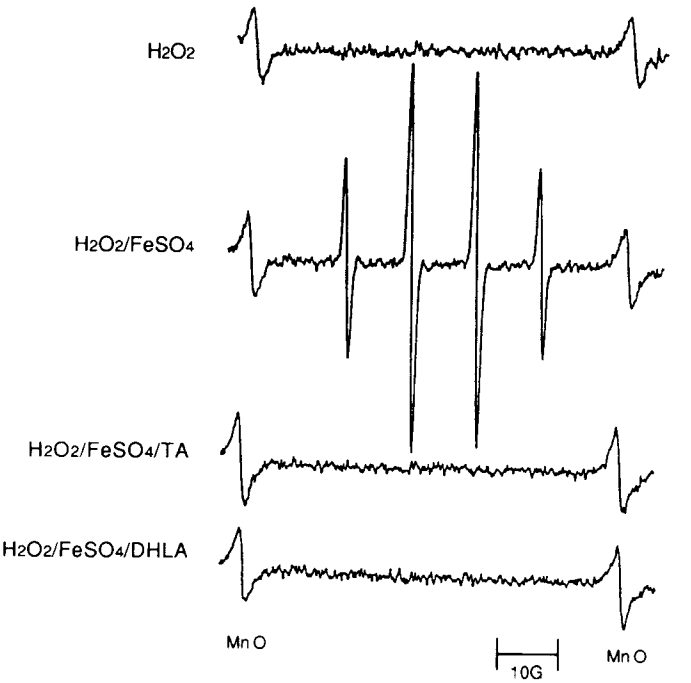

FIGURE 2 ESR study of the effects of thioctic acid and dihydrolipoic acid on hydroxyl radicals generated by hydrogen peroxide plus ferrous sulfate. DMPO spin adducts generated by 2 mM hydrogen peroxide plus 0.2 mM ferrous sulfate in 150 mM KH_2PO_4-KOH (pH 7.4) in total volume of 1 ml. [DMPO] = 1 mM; [thioctic acid (TA)] = 1 mM; [dihydrolipoic acid (DHLA)] = 1 mM. ESR signals were recorded 3 min after the initiation of Fenton reaction.

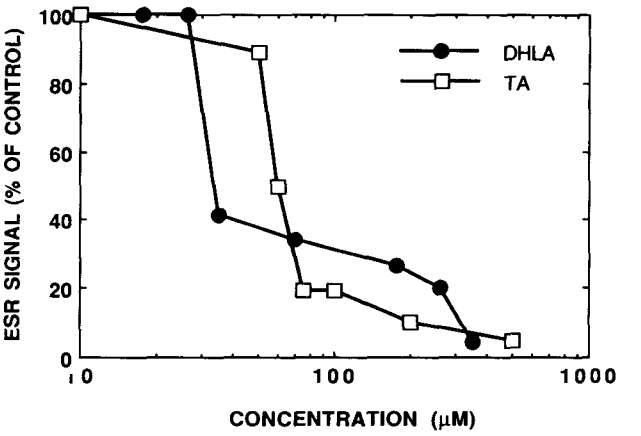

FIGURE 3 Concentration dependence of thioctic acid and dihydrolipoic acid on hydroxyl radicals generated by hydrogen peroxide plus ferrous sulfate.

by 0.5 mM TA or DHLA. Figure 3 shows that concentration dependent inhibition of the Fenton reaction by TA and DHLA. These agents at 0.2 mM were capable of completely eliminating the DMPO–OH which was generated by 0.2 mM $FeSO_4$ + 2 mM H_2O_2 (approximately 1 : 1 stoichiometry ratio for TA or DHLA to the possibly generated HO · concentration).

To confirm a finding that TA is capable of acting as an antioxidant, a chemiluminescence study was conducted using luminol as a probe for HO ·. As shown in Figure 4, TA (0.5 mM) significantly inhibited the generation of the chemiluminescence product of HO · generated by 20 μM H_2O_2 and 1 μM $FeSO_4$ (down to 27.7 $\pm$ 7.1% of control).

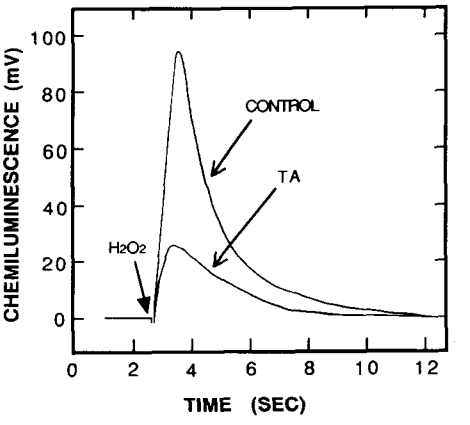

FIGURE 4 Chemiluminescence study of the effects of thioctic acid on hydroxyl radicals generated by hydrogen peroxide plus ferrous sulfate. Hydroxyl radicals were detected using luminol as a probe in 150 mM KH_2PO_4-KOH (pH 7.4) at room temperature in total volume of 2.0 ml while vortexing. [luminol] = 2.5 μM; [$FeSO_4$] = 1 μM; [H_2O_2] = 20 μM.

A possible mechanism of the inhibition of the Fenton reaction by TA may be that Fe^{2+} reduces TA to DHLA

$$TA + Fe^{2+} \rightarrow DHLA + Fe^{3+}.$$

Thus, TA, in effect, may act as a scavenger of Fe^{2+} which is necessary for Fenton reaction. Also the subsequently generated DHLA may act as a scavenger for HO·:

$$DHLA + HO\cdot \rightarrow \text{products}.$$

This hypothesis was tested by examining whether TA is capable of oxidizing Fe^{2+} to Fe^{3+}, resulting in a decrease of Fe^{2+} detected by the *o*-phenanthroline assay. The hypothesis, however, was not validated by this methodology as the results showed that 1 mM TA was not able to compete with 200 μM *o*-phenanthroline for 100 μM Fe^{2+} at pH 7.4 (data not shown).

Instead of the reduction of TA by Fe^{2+}, its converse, the reduction of Fe^{3+} by DHLA has been suggested in the literature:

$$DHLA + Fe^{3+} \rightarrow TA + Fe^{2+}.$$

The implication is that DHLA can be a prooxidant.[3] This was also tested using the phenanthroline assay. This effect was not validated because we did not observe an increase in Fe^{2+}-phenanthroline complex formation induced by the reduction of 100 μM Fe^{3+} by up to 5 mM of DHLA at pH 7.4 (data not shown). A further absence of a prooxidant activity of DHLA was also observed by an ESR. H_2O_2 (2 mM) plus ferric sulfate (0.2 mM) did not generate DMPO–OH adduct as expected, and the addition of 0.5 mM DHLA did not generate DMPO–OH adduct in this system (pH 7.4) suggesting that DHLA does not potentiate the generation of HO· by Fenton's reagent (at least the prooxidant activity of DHLA did not overcome its antioxidant activity).

DISCUSSION

Although their roles as membrane antioxidants have already been found to be important, the findings reported here that TA and DHLA possess the antioxidant properties in the aqueous phase may add to their effectiveness.

The antioxidant activity of DHLA against O_2^- appears to involve the $-SH$ groups of DHLA. The transfer of an electron from DHLA to O_2^- is strongly suggested from the observation of the formation of H_2O_2. Oxidation of DHLA by O_2^- does not seem to form thiyl radicals as shown in the bottom spectrum of Figure 1 (no peaks were observed). The reaction of DHLA with O_2^- may have considerable significance in contribution to its ability to act as an effective antioxidant because O_2^- is generally known to be the first radical formed in the biological systems and the elimination of this radical must be a very effective way of defending against free radical toxicity.

We also observed that both TA and DHLA are capable of eliminating the HO· generated by the Fenton's reagent. HO· can react with most organic compounds with a high second order kinetic rate constants ranging from 10^7–10^{10} $M^{-1}sec^{-1}$.[15] Thus, it is not very surprising that excess of any organic compound would eliminate the HO·. However, in case of both TA and DHLA, they eliminate the HO· with stoichiometry ratios between TA/DHLA and HO· very close to 1 : 1. This suggests that they possess very high reactivity against the HO· which would allow to overcome

the competition with DMPO. Our earlier findings in this laboratory suggested that TA had no ability in inhibiting lipid peroxidation induction by 1,1′-azobis(2,4-dimethylvaleronitrile), AMVN.[6] AMVN, however, is the initiator of peroxyl radical, and TA may react with the HO · and may not react with the peroxyl radical. Alternatively, a possible mechanism may be that TA behaves differently in the membranes as it does in the aqueous phase as an antioxidant.

Another possible mechanism of inhibition of Fenton reaction is the chelation of iron ions. DHLA has been found to bind Fe^{3+}.[16] Our results using *o*-phenanthroline, however, have shown that there is no interactions between TA/DHLA and iron ions, at least, in terms of electron transfer. The high affinity of *o*-phenanthroline to ferrous ion,[10] however, may preclude us from detecting the observable effects of TA and DHLA on iron ions using this methodology. In relation to the effects of iron ions, our data also disagree with findings by Bast and Haenen[3] that DHLA by itself acts as a prooxidant which potentiates lipid peroxidation of the membrane (by reducing iron ions), and it only can act as an antioxidant in the presence of glutathione. This again may reflect onto the possible difference in the activities of TA/DHLA in different phases of the biological system (i.e., membrane and aqueous phases). Although further studies are needed to elucidate the exact mechanism, TA and DHLA possess antioxidant properties against the HO · generated by the Fenton reagents.

In conclusion, the results from present study suggest that TA/DHLA redox couple seems promising in a biological antioxidant activity.

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