BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **227,** 216–220 (1996)
ARTICLE NO. 1492

Reevaluation of Superoxide Scavenging Activity of Dihydrolipoic Acid and Its Analogues by Chemiluminescent Method Using 2-Methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo-[1,2-*a*]pyrazine-3-one (MCLA) as a Superoxide Probe

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Received June 30, 1996

The superoxide scavenging activity of dihydrolipoic acid and its analogues has been reevaluated by a chemiluminescence method using MCLA as a superoxide indicator. The results demonstrated that all the compounds having the thiol chromophore in the molecule showed scavenging activity toward superoxide. The short-chain analogue, tetranor-dihydrolipoic acid, showed almost the same scavenging activity as that of dihydrolipoic acid; however, bisnor-dihydrolipoic acid showed weaker scavenging activity than that of dihydrolipoic acid. The reaction rates of dihydrolipoic acid, bisnor-dihydrolipoic acid, and tetranor-dihydrolipoic acid were calculated from the competitive inhibition of MCLA-superoxide reaction. Thus, it was concluded that dihydrolipoic acid and the analogues are good scavengers of superoxide radical anion.

INTRODUCTION

Recently, a great deal of attention has been given to the antioxidant activity of α-lipoic acid (LA) and its reduced form, dihydrolipoic acid (DHLA) (Fig. 1) [1-4]. As for the antioxidant activity of DHLA toward superoxide, there is a controversy about whether DHLA acts as a scavenger for superoxide or not [5, 6]. Two groups (Suzuki *et. al.* [5] and Scott *et. al.* [6]) reported opposite results that were based upon ESR and nitro-bluetetrazolium (NBT) experiments, respectively. However, we anticipated that the high reducing ability of the SH group of reduced LA will disturb the measurement of superoxide mediated reduction. So, it is highly desiable not to use a compound like NBT as a reporter molecule. In order to exclude this ambiguity, we applied a chemiluminescence method developed by Nakano *et al.* [7], because MCLA chemiluminescence is a highly sensitive and specific indicator for superoxide, and further, the chemiluminescent MCLA-O_2 complex is stable and non-reactive toward DHLA analogues bearing the highly reducing sulfhydryl group. In this communication, the superoxide scavenging activity of DHLA and its short-chain analogues, bisnor-dihydrolipoic acid and tetranor-dihydrolipoic acid together with cysteine and dithiothreitol were also evaluated.

MATERIALS AND METHODS

Xanthine, xanthine oxidase and superoxide dismutase were obtained from the Sigma Chemical Co. Ltd. MCLA was from Tokyo Chemical Industry (TCI). Other reagents were of reagent grade and used without further purification except where indicated. Bisnor-dihydrolipoic acid and tetranor-dihydrolipoic acid were prepared by the same method as described previously [8]. DHLA, bisnor-dihydrolipoic acid and tetranor-dihydrolipoic acid (ASTA Medica) were

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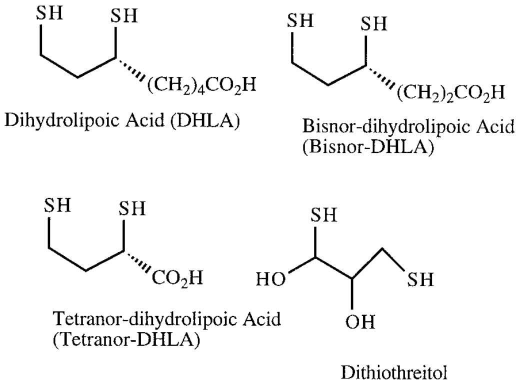

FIG. 1. Structure of bisnor, tetranor, and dihydrolipoic acid.

dissolved in ethyl alcohol at defined concentrations and were kept at 0°C before use to avoid oxidation. Chemiluminescence spectra were measured using a Perkin-Elmer MPF-44A fluorescence spectrophotometer.

MCLA chemiluminescence measurement. The chemiluminescence emission due to the reaction of superoxide and MCLA was measured at 465 nm after excitation at 350 nm using a Perkin-Elmer MPF-44A fluorescence spectrometer. The scavenging activity was determined either kinetically or by steady state inhibition of growth of the chemiluminescence signal. For the kinetic method, 5 μl of the MCLA aqueous solution (500 μg / ml) was added to 1.8 ml of buffer, 50 mM Na carbonate containing 50 mM Xanthine at pH 7.5 in 3 ml fluorescence cuvette. After baseline chemiluminescence was traced for 1 min, then, 1 unit of xanthine oxidase was added under vigorous shaking to generate superoxide anion. After recording the growth of the chemiluminescence for 1 min, an aliquot of scavengers were added to observe the slope change of the growth curve. For the steady state inhibition study, the compound was added first to the reaction solution and then xanthine oxidase was added to initiate the reaction. The steady state chemiluminescence intensity (at 2 min after xanthine oxidase addition) was measured to evaluate the scavenging activity. All the reactions were carried out at room temperature.

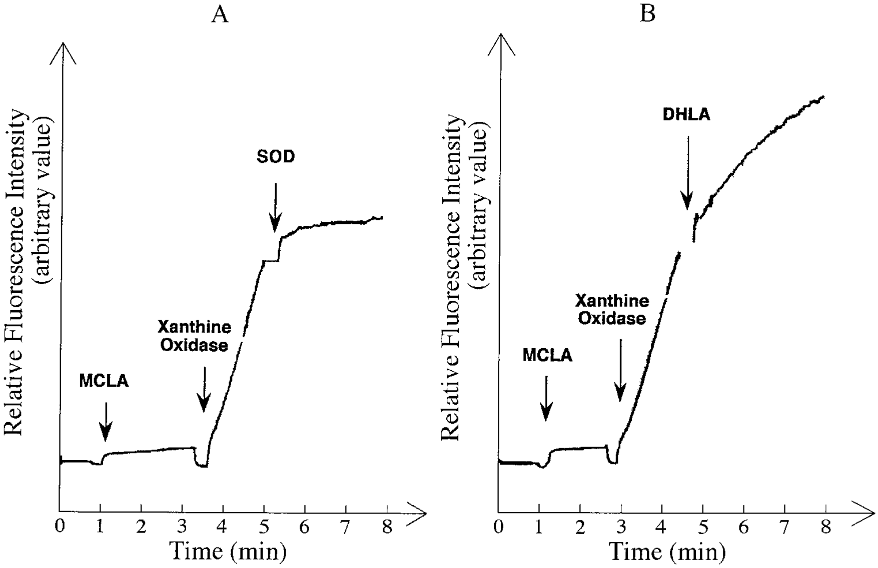

FIG. 2. Typical traces of chemiluminescence signals due to the MCLA-superoxide reaction. Chemiluminescence was measured at 465 nm emission after excitation at 350 nm. Reagents were added at the point indicated by the arrow. (A) Effect of SOD, (B) Effect of DHLA.

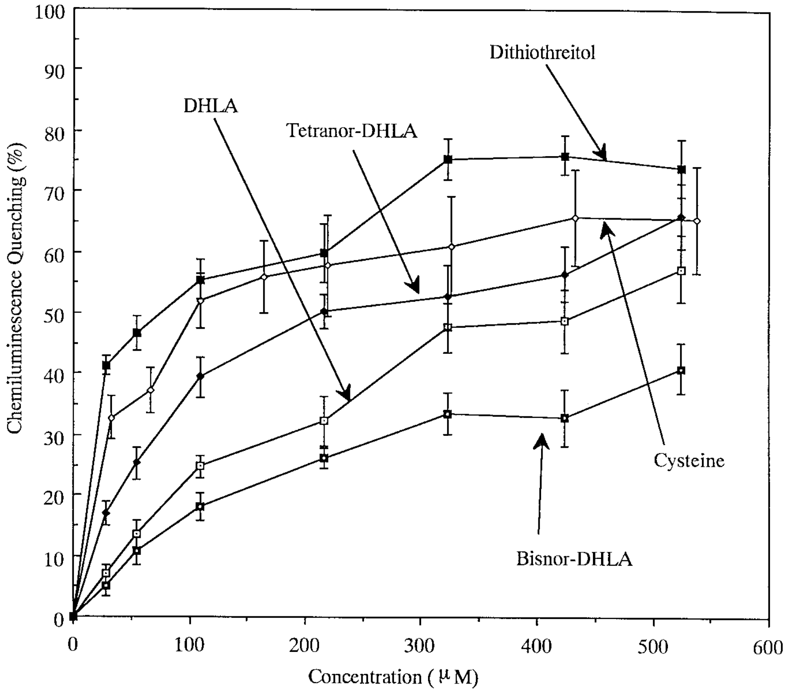

FIG. 3. Concentration dependence of superoxide scavenging activity of thiol compounds. Reaction conditions were described in the Materials and Methods. Scavengers added prior to the initiation of chemiluminescence by xanthine oxidase and the scavenging activity was determined from the chemiluminescence intensity 2 min after xanthine oxidase addition. Values are the mean of triplicate experiments.

RESULTS

When xanthine oxidase was added to the reaction mixture containing xanthine and MCLA, superoxide generation takes place due to the reaction of MCLA with superoxide. The chemiluminescence growth is completely inhibited by the addition of superoxide dismutase (SOD) to this system. DHLA also markedly inhibited the chemiluminescence indicating superoxide scavenging activity of DHLA (Fig. 2). This is also the case for short-chain analogues of DHLA such as bisnor- and tetranor-dihydrolipoic acid. The concentration dependence of their

TABLE I
Reaction Rates of Thiol Containing Compounds toward Superoxide

Compound	Reaction rate $(M^{-1} \cdot s^{-1}) \times 10^5$
DHLA	1.43
Bisnor-DHLA	0.72
Tetranor-DHLA	2.55
Cysteine	5.00
Dithiothreittol	7.14
DHLA[a]	3.30

[a] Determined from the reaction using epinephrine [ref. 5].

inhibitory activity were determined and the results are summarized in Fig. 3. Superoxide scavenging activity was the strongest for dithiothreitol followed by cysteine, teranor-dihydrolipoic acid and DHLA. Bisnor-dihydrolipoic acid showed the weakest activity among the sulfhydryl compounds tested. Based on the data of the steady state inhibition of MCLA chemiluminescence, we calculated the bimolecular reaction rates for these compounds. From the data in the fig. 3, the concentration for 50% inhibition for these thiol compounds were as follows: cysteine 108 μM, tetranor-DHLA 210 μM, DHLA 375 μM, bisnor-DHLA 748 μM and dithiothreitol 75 μM. Since the MCLA concentration was 4.7 μM, the bimolecular rate constant for the scavenging reaction can be shown as follows:

$$\text{(i)}\quad k_{\text{cys}} = \frac{4.7}{108} \times k_{\text{MCLA}}$$

$$\text{(ii)}\quad k_{\text{DHLA}} = \frac{4.7}{375} \times k_{\text{MCLA}}$$

$$\text{(iii)}\quad k_{\text{bisnor-DHLA}} = \frac{4.7}{748} \times k_{\text{MCLA}}$$

$$\text{(iv)}\quad k_{\text{tetranor-DHLA}} = \frac{4.7}{210} \times k_{\text{MCLA}}$$

$$\text{(v)}\quad k_{\text{dithiothreitol}} = \frac{4.7}{75} \times k_{\text{MCLA}}$$

Using the reported k_{cys} value as $5 \times 10^{5}\ M^{-1} \cdot s^{-1}$ [9], the k_{MCLA} toward superoxide was calculated as 1.14×10^{7}. This allowed us to calculate the rate constants for the DHLA analogues as shown in Table I.

DISCUSSION

Present studies using MCLA as a reporter molecule for superoxide clearly demonstrated that DHLA and its analogues have comparable superoxide scavenging activity as that of cysteine. There was, however, no direct relation between the alkyl chain length and the superoxide scavenging activity of the three DHLA analogues. The reactivity of DHLA toward superoxide is controversal. In our laboratory Suzuki *et al.* first reported that DHLA is a good scavenger toward superoxide using ESR spin trapping techniques. We reported a second order rate constant of the DHLA reaction toward superoxide as $3.3 \times 10^{5}\ M^{-1}s^{-1}$. This value was obtained by the competition reaction of DHLA with epinephrine [5]. This value strongly correlates with our present results using chemiluminescent MCLA as the molecular reporter. On the other hand, in 1994 Scott *et al.* arrived at completely different conclusion, namely that DHLA does not scavenge superoxide from their NBT reduction experiments [6]. Thus, the conclusion drawn were completely opposite although the superoxide generation system used by both groups was the same. We followed the same experiments as reported by Scott *et al.* using NBT, but found that DHLA reacts directly with NBT and reduces it before superoxide reacts with NBT (results not shown). Therefore, the superoxide dependent reduction of NBT becomes artificially small at higher DHLA concentrations which may have lead to an erroneous conclusion. Indeed, we observed that an excess amount of DHLA completely reduced NBT within 30 seconds. In contrast to NBT, MCLA was found to be quite stable against sulfhydryl bearing compounds with high reducing potential. Furthermore, the chemiluminescent reaction products of MCLA with superoxide were stable and were not affected by DHLA. It is thus concluded that the observed chemiluminescence quenching by DHLA is due to the superoxide scavenging activity of DHLA.

ACKNOWLEDGMENT

Supported by NIH Grant # 1 RO1 DK 50430-01A1.

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