

The antioxidant action of 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2- α]pyrazin-3-one (MCLA), a chemiluminescence probe to detect superoxide anions

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Abstract The antioxidant effect of 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2- α]pyrazin-3-one (MCLA), a *Cypridina* luciferin analog that acts as a chemiluminescence probe to detect $O_2^{\cdot-}$, was investigated. MCLA produced a lag in oxygen consumption induced by cumene hydroperoxide in microsomes or by 2,2'-azobis (2-amidinopropane) dihydrochloride in liposomes and disappeared during the duration of the lag. MCLA profoundly inhibited the propagation reaction in Fe^{2+} -dependent lipid peroxidation in liposomes, and MCLA disappearance accompanied by suppression of oxygen consumption markedly occurred in liposomes susceptible to peroxidation. Thiobarbituric acid-reactive substances in all systems used were also suppressed by MCLA dose dependently. These results indicate that MCLA has an antioxidant property through scavenging free radicals.

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Key words: 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2- α]pyrazin-3-one; Chemiluminescence; Lipid peroxidation; Antioxidant; Superoxide anion; Free radical

1. Introduction

A *Cypridina* luciferin analog, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2- α]pyrazin-3-one (MCLA), is an excellent chemiluminescence probe to detect $O_2^{\cdot-}$, in both its specificity and selectivity [1]. Fig. 1 shows the structure of MCLA, which has a nucleus of 3,7-dihydroimidazo[1,2- α]pyrazin-3-one that is important to react with $O_2^{\cdot-}$. Luminescence of MCLA involves reaction of MCLA with $O_2^{\cdot-}$ to yield an unstable dioxetane, whose decomposition emits light [1]. Thus, MCLA has been used to detect $O_2^{\cdot-}$ generated by a variety of biological materials, including leukocytes and macrophages [1–4]. Koga and Nakano [5] reported that MCLA, as well as copper salicylate that is a superoxide dismutase mimetic, inhibits NADPH-supported lipid peroxidation in microsomes. In their study, superoxide dismutase (SOD) completely nullified MCLA luminescence, but the peroxidation was not inhibited by the enzyme. They proposed, therefore, that the inhibitory action of MCLA arises from the MCLA scavenging $O_2^{\cdot-}$ generated from the cytochrome P450 system

at intramembranous sites, leading to suppressing $O_2^{\cdot-}$ -dependent iron reduction. Indeed, cytochrome P450 functions as an $O_2^{\cdot-}$ generator, resulting in lipid peroxidation, in the reconstituted peroxidation system, including NADPH-cytochrome P450 reductase [6]. Afanas'ev et al. [7] also proposed that $O_2^{\cdot-}$ generated in the lipid phase is an obligatory initiation species in NADPH-supported lipid peroxidation in microsomes.

Whether the inhibitory effect of MCLA on lipid peroxidation is really due to removing $O_2^{\cdot-}$ or whether MCLA itself exerts an antioxidant effect seems important. However, the antioxidant effect of MCLA in $O_2^{\cdot-}$ -independent systems has not been determined. In this study, we examined the antioxidant activity of MCLA on iron-independent and iron-dependent lipid peroxidation in the systems without $O_2^{\cdot-}$ involvement. We show here that MCLA has the ability to scavenge free radicals.

2. Materials and methods

2.1. Materials

MCLA was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). L- α -phosphatidylcholine β -arachidonoyl γ -stearoyl (PC(A)), L- α -phosphatidylcholine dipalmitoyl (PC(DP)), DL- α -phosphatidyl-L-serine dipalmitoyl (PS) and SOD from bovine erythrocytes were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ferrous pyrophosphate (Fe^{2+} -PPi, 1:0.9) and ferric pyrophosphate (Fe^{3+} -PPi, 1:0.9) were prepared as we described previously [8]. All other chemicals used were commercial reagent grade quality. Water processed with a MilliQ system (Millipore) was used to prepare all reagents.

2.2. Preparation of microsomes

Male Sprague-Dawley rats (320 to 340 g) were killed by decapitation and the livers were perfused with 0.9% NaCl, were removed, and were homogenized in 5 volumes of 0.25 M sucrose. The microsomal pellets obtained by the conventional centrifugation method were washed twice with 0.15 M KCl containing 10 mM Tris-HCl (pH 7.5), and were resuspended finally in 50 mM Tris-HCl (pH 7.5). Microsomal protein was measured by the biuret method [9].

2.3. Preparation of liposomes

PC(A), which has arachidonic acid at the β -position, was used as the substrate of lipid peroxidation, and PS, which has no unsaturated fatty acid, was used to obtain the negative charge on the surface of the liposomes. Unilamellar liposomes were prepared by sonication as we described previously [8]. All operations were carried out under argon and sonication was performed in ice-cold water. The hydroperoxide contents in liposomes were determined iodometrically by the method of Buege and Aust [10].

2.4. Lipid peroxidation

The lipid peroxidation in the microsomes (0.5 mg protein/ml) was initiated with 0.3 mM cumene hydroperoxide (CHP) in 0.15 M KCl and 25 mM Tris-HCl (pH 7.5). The incubation mixture for the NADPH-supported system contained cholate-solubilized microsomes

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Abbreviations: AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; CHP, cumene hydroperoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; MCLA, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2- α]pyrazin-3-one; MDA, malondialdehyde; PC(A), L- α -phosphatidylcholine β -arachidonoyl γ -stearoyl; PC(DP), L- α -phosphatidylcholine dipalmitoyl; PPi, pyrophosphate; PS, DL- α -phosphatidyl-L-serine dipalmitoyl; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances

(0.5 mg protein/ml), 10 μM Fe^{3+} -ADP (1:17) and 0.4 mM NADPH. Microsomes (20 mg protein/ml) were solubilized with 1.2% sodium cholate as we described previously [11]. Lipid peroxidation in liposomes was initiated with Fe^{2+} -PPI or 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). The incubation system consisted of 0.2 mM PC(A)/40 μM PS liposomes, 40 μM Fe^{2+} -PPI or 50 mM AAPH, 0.15 M KCl and 25 mM Tris-HCl (pH 7.5). Oxygen consumption was measured in a 1.5 ml cell at 37°C with a Clark electrode in a YSI Model 5300 Biological Oxygen Monitor (Yellow Springs, OH, USA). Thiobarbituric acid-reactive substances (TBARS) were quantified by the method of Ottolenghi [12] using an extinction coefficient at 535 nm of $156 \text{ mM}^{-1} \text{ cm}^{-1}$, and the result was expressed in terms of malondialdehyde (MDA) production. The background activity of MDA production before incubation was subtracted in each experiment.

2.5. Measurement of free radical scavenging ability

The peroxyl radical scavenging ability of MCLA was compared with Trolox by monitoring oxygen consumption in AAPH-supported liposomes. The efficiency of the radical scavenging of the antioxidant was calculated as the ratio of the rate constant for inhibition of oxidation (k_{inh}) to the rate constant for chain propagation (k_p) [13]:

$$k_{\text{inh}}/k_p = [\text{LH}]/R_{\text{inh}}t_{\text{inh}}$$

where LH is the concentration of PC(A); R_{inh} is the rate of oxidation during the inhibition period (t_{inh}). The stoichiometric factor (n) for peroxyl radical trapping by MCLA was obtained from the following equation [14]:

$$n = R_{\text{t}}t_{\text{inh}}/[\text{IH}]$$

where IH is the concentration of antioxidant; R_{t} is the rate of initiation. For the reactivity of MCLA with 1,1-diphenyl-2-picrylhydrazyl (DPPH), 5 to 25 μM MCLA in ethanol was mixed with 100 μM DPPH in ethanol, and the mixtures were incubated at 25°C. Because the reaction was completed within 10 s, DPPH disappearance at 517 nm [15] was determined after 10 s incubation.

2.6. Other assays

Disappearance of MCLA was monitored by recording a decrease in absorbance at 430 nm at 37°C. The reaction under anaerobic conditions was made by purging the incubation mixture with argon using a screw-topped quartz cell. Oxidation of NADPH was monitored at 340 nm at 37°C.

3. Results

The effect of MCLA on CHP-supported lipid peroxidation in microsomes, which is mediated by the cumoxyl radical ($\text{CumO}^\bullet$) as the main species [16,17], was examined (Fig. 2). MCLA completely suppressed oxygen consumption in the early stage, which is referred to as a lag phase. The lag was prolonged by increasing the concentrations of MCLA from 5 to 25 μM (Fig. 2A). MCLA inhibited CHP-supported lipid peroxidation concentration dependently in terms of both oxygen consumption and TBARS production. In the system including microsomes, MCLA at 25 μM , a concentration that caused almost complete inhibition of the lipid peroxidation

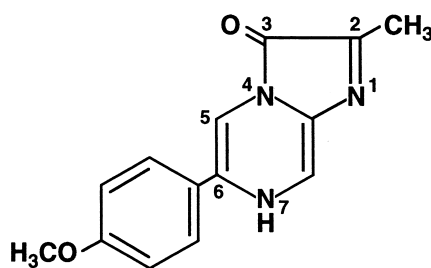


Fig. 1. The chemical structure of MCLA.

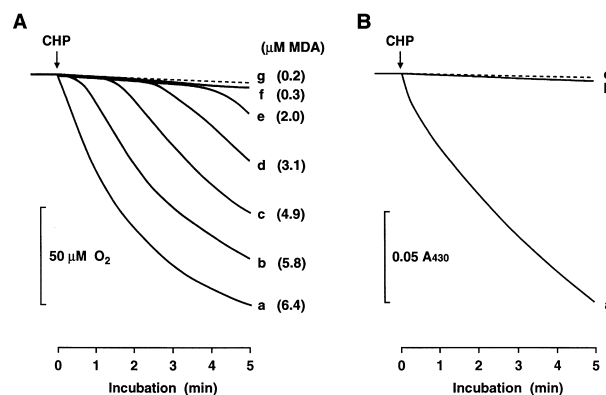


Fig. 2. Effect of MCLA on CHP-supported lipid peroxidation (A) and MCLA disappearance (B) in microsomes. The reaction system consisted of microsomes (0.5 mg protein/ml), 0.3 mM CHP, 0.15 M KCl, and 25 mM Tris-HCl (pH 7.5). In A, the concentrations of MCLA added were: a, 0 μM ; b, 5 μM ; c, 10 μM ; d, 15 μM ; e, 20 μM ; f, 25 μM . Dotted line g indicates oxygen consumption with microsomes alone. The figures in parentheses represent TBARS production after 6 min incubation. MCLA disappearance (B) was monitored in the presence of MCLA at a fixed concentration of 25 μM . a, with CHP; b, without CHP. Dotted line c indicates MCLA disappearance with CHP in the absence of microsomes. The addition of CHP is indicated by an arrow. Data represent at least three independent experiments, which showed similar results.

(Fig. 2A, curve f), dramatically disappeared in the presence of CHP, but the disappearance did not occur in the absence of either CHP or microsomes (Fig. 2B).

In the course of the experiments, we confirmed that MCLA did not affect NADPH oxidation during NADPH- and iron-supported lipid peroxidation in microsomes (data not shown). The finding shows that MCLA appears not to inhibit the reduction of iron responsible for the peroxidation. Additionally, to exclude completely any nonheme iron from interaction with MCLA, CHP-supported lipid peroxidation was tested in the presence of desferal (deferrioxamine mesylate), an iron

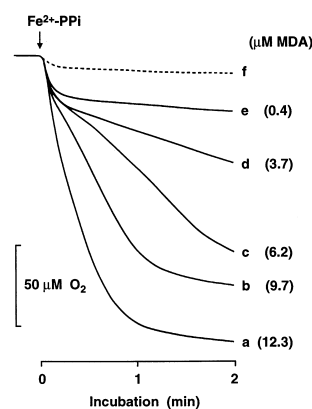


Fig. 3. Effect of MCLA on Fe^{2+} -supported lipid peroxidation in phospholipid liposomes. The incubation mixtures contained 0.2 mM PC(A)/40 μM PS liposomes, including hydroperoxide (6 μM), 40 μM Fe^{2+} -PPI, 0.15 M KCl, and 25 mM Tris-HCl (pH 7.5). The concentrations of MCLA added were: a, 0 μM ; b, 5 μM ; c, 7.5 μM ; d, 10 μM ; e, 20 μM . Dotted line f indicates oxygen consumption in Fe^{2+} autooxidation in the absence of both MCLA and liposomes. The figures in parentheses represent TBARS production after 3 min incubation. The addition of Fe^{2+} is indicated by an arrow. Data represent at least three independent experiments, which showed similar results.

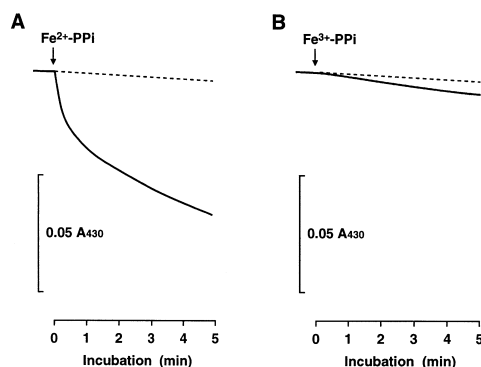


Fig. 4. MCLA disappearance in Fe^{2+} - (A) or Fe^{3+} - (B) supported systems containing phospholipid liposomes. The incubation mixtures contained liposomes, $40 \mu\text{M}$ Fe^{2+} -PPi (A) or $40 \mu\text{M}$ Fe^{3+} -PPi (B), $20 \mu\text{M}$ MCLA, 0.15 M KCl, and 25 mM Tris-HCl (pH 7.5). Solid lines represent 0.2 mM PC(A)/ $40 \mu\text{M}$ PS liposomes, including hydroperoxide ($6 \mu\text{M}$); dotted lines represent 0.2 mM PC(DP)/ $40 \mu\text{M}$ PS liposomes. Data represent at least two independent experiments, which showed similar results.

chelator, which is also reported to be a peroxy radical scavenger [18]. Although desferal (0.1 mM) suppressed approximately 15% of the peroxidation, the inhibitory profiles of oxygen consumption with MCLA were almost the same as those in the absence of desferal (data not shown). This result implies that iron, which may be present in microsomes, does not interfere with the antioxidant action of MCLA.

The effect of MCLA on Fe^{2+} -supported lipid peroxidation

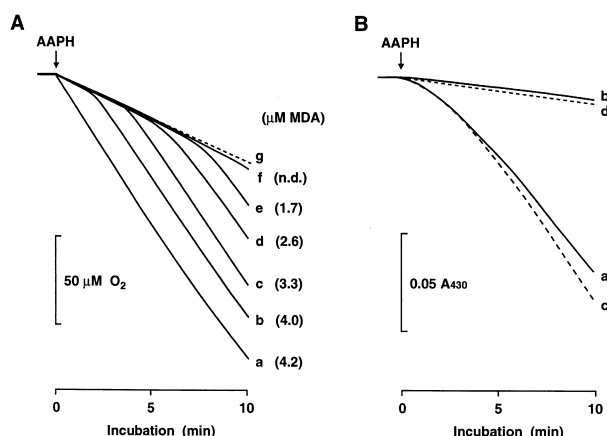


Fig. 5. Effect of MCLA on AAPH-induced lipid peroxidation (A) and MCLA disappearance (B) in liposomes. The reaction system consisted of 0.2 mM PC(A)/ $40 \mu\text{M}$ PS liposomes, 50 mM AAPH, 0.15 M KCl, and 25 mM Tris-HCl (pH 7.5). In A, the concentrations of MCLA added were: a, $0 \mu\text{M}$; b, $5 \mu\text{M}$; c, $10 \mu\text{M}$; d, $15 \mu\text{M}$; e, $20 \mu\text{M}$; f, $25 \mu\text{M}$. Dotted line g indicates oxygen consumption with AAPH alone or with both AAPH and MCLA ($25 \mu\text{M}$) in the absence of liposomes, respectively. The figures in parentheses represent TBARS production after 12 min incubation. MCLA disappearance (B) was monitored in the presence of MCLA at a fixed concentration of $25 \mu\text{M}$. a, with AAPH; b, without AAPH. Dotted lines c and d indicate MCLA disappearance with AAPH in the absence of liposomes under air and under argon, respectively. The addition of AAPH is indicated by an arrow. n.d. denotes 'not detected'. Data represent at least three independent experiments, which showed similar results.

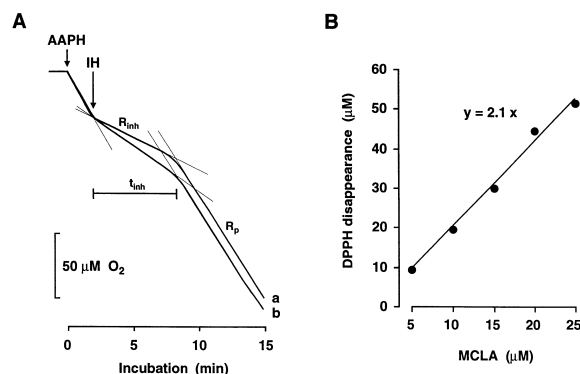


Fig. 6. Free radical scavenging ability of MCLA on peroxy radical (A) and DPPH (B). The composition of incubation mixtures for lipid peroxidation in AAPH-supported liposomes (A) was the same as in Fig. 5, except that the concentration of both MCLA and Trolox was $15 \mu\text{M}$. Each antioxidant (IH) was added 2 min after the reaction was initiated with AAPH. Abbreviations of R_{inh} , R_p , and t_{inh} are the rate of oxidation during the inhibition period, the rate of propagation, and the inhibition period, respectively. Added were: a, MCLA; b, Trolox. DPPH disappearance (B) based on reaction with MCLA was determined as described in Section 2.

in liposomes was examined (Fig. 3). To exclude the possible involvement of O_2^- produced from Fe^{2+} autoxidation, we checked that SOD (100 units/ml) had no influence on the peroxidation (data not shown). As indicated by the initial drop in each curve of oxygen consumption just after adding Fe^{2+} -PPi, MCLA showed no inhibition of oxygen consumption at first, regardless of its concentrations. Thereafter, however, MCLA inhibited the propagation reaction dose dependently in Fe^{2+} -supported lipid peroxidation, monitored by measuring oxygen consumption. Dose-related inhibitions of MCLA were also demonstrated by TBARS production. The rates of MCLA disappearance after adding Fe^{2+} -PPi were much higher in PC(A)/PS liposomes having arachidonate than in PC(DP)/PS liposomes without unsaturated fatty acids (Fig. 4A), while the rate with Fe^{3+} -PPi was very low, even in PC(A)/PS liposomes (Fig. 4B).

AAPH thermally decomposes to 2-amidinopropane radicals ($\text{A}\cdot$), followed by instantaneous reaction with oxygen to give peroxy radicals ($\text{AOO}\cdot$), which are capable of initiating lipid peroxidation [19]. MCLA inhibited AAPH-induced lipid peroxidation in liposomes dose dependently in terms of oxygen consumption and TBARS production (Fig. 5A). The inhibitory profiles of oxygen consumption by MCLA in the early stages were similar to those in the CHP-supported system; again MCLA produced a lag, which was prolonged by increasing the concentrations. Without liposomes, MCLA had no effect on AAPH-dependent oxygen consumption, compared with AAPH alone. Alternatively, MCLA disappearance was markedly caused by adding AAPH in the presence or the absence of liposomes under aerobic conditions (Fig. 5B). The disappearance was completely nullified under argon.

The peroxy radical scavenging ability of MCLA in AAPH-supported liposomes was compared with that of Trolox (Fig. 6A). The inhibition periods (t_{inh}) of these antioxidants were almost equal (MCLA, 6.4 min ; Trolox, 6.3 min). The k_{inh}/k_p of MCLA and Trolox were calculated to be 6.5 and 4.6 , respectively. These values show that the free radical scavenging ability of MCLA is slightly more effective than that of

Trolox. The stoichiometric factor (n) for peroxy radical trapping for MCLA was 2, in agreement with Trolox ($n = 2$) [14]. DPPH disappeared linearly with the increase in MCLA concentrations and one mole of MCLA reacted with 2 moles of the radical (Fig. 6B).

4. Discussion

MCLA showed no inhibition in oxygen consumption initially after adding Fe^{2+} -PPi to PC(A)/PS liposomes, which contained traces of lipid hydroperoxides (6 μM), although MCLA profoundly inhibited the subsequent propagation reaction. MCLA at 20 μM , a concentration that caused almost complete inhibition of the propagation, markedly disappeared in the propagation stage. These results suggest that MCLA is capable of scavenging lipid peroxy radicals, as described below. Lipid hydroperoxide-dependent reactions are catalyzed by Fe^{2+} -dependent decomposition of lipid hydroperoxides, leading to chain reactions by alkoxy radical generation. However, Wilcox and Marnett [20] demonstrated that lipid alkoxy radicals derived from the decomposition of conjugated dieny hydroperoxides by Fe^{2+} do not exist as reactive intermediates, because the intramolecular cyclization of lipid alkoxy radicals into the adjacent site of unsaturation is instantaneous. Subsequent consumption of one mole of oxygen, coupled with loss of conjugated dienes, results in the generation of epoxy peroxy radicals as reactive intermediates [21]. We previously reported that oxygen consumption observed initially after adding iron was dependent on the hydroperoxide content of liposomes, and that even butylated hydroxytoluene, a potent radical scavenger, incorporated into liposomes could not prevent the oxygen consumption, whereas the propagation reaction was completely inhibited [22]. In this study, therefore, MCLA would not be consumed initially after adding Fe^{2+} -PPi; until consumption starts molecular oxygen equivalent to hydroperoxide present in liposomes would be taken up. Thereafter, MCLA possibly reacts with lipid peroxy radicals.

MCLA produced a lag in CHP-supported lipid peroxidation accompanied by MCLA disappearance in microsomes. These observations suggest that MCLA reacts with $\text{CumO}\cdot$ before $\text{LOO}\cdot$ production from phospholipids following hydrogen abstraction. In AAPH-induced lipid peroxidation in liposomes, MCLA also produced a lag accompanied by MCLA disappearance. From the differences between MCLA disappearance in the absence of liposomes under air and under argon, MCLA reacted with $\text{AOO}\cdot$, but did not react with $\text{A}\cdot$ under the conditions.

The free radical scavenging ability of MCLA was slightly higher than that of Trolox. The stoichiometry for MCLA trapping either peroxy radical or DPPH was 2, as expected from the equal t_{inh} of both antioxidants. MCLA has no similarity of the partial structures to known free radical scavengers [18]. However, from the reactivity with $\text{O}_2^{\cdot-}$ [1] and the inhibition of peroxidation by the 6-phenyl congener of MCLA [5], the structure essential for the antioxidant action is presumed to be 3,7-dihydroimidazo[1,2- α]pyrazin-3-one. In support of this, Goto [23] suggested that a reaction of a hydroperoxy radical generated from hydrogen peroxide and ferricyanide (at pH 5.6) with a *Cypridina* luciferin analogue yields its hydroperoxide derivatives. Similarly, if the radical scavenging action of MCLA on $\text{LOO}\cdot$ yields MCLA-OOL, two moles of $\text{LOO}\cdot$ could be consumed. Reactions of peroxy

radical with MCLA may be possible: a hydrogen abstraction from $\text{H-N} <$ (position 7) and the subsequent peroxy radical binding following the radical rearrangement. Similar to the action of antioxidant phenol compounds, the fact seems plausible that the antioxidant action of MCLA donates a hydrogen atom and thereby converts the peroxy radical to hydroperoxide.

MCLA has the latent ability to scavenge $\text{O}_2^{\cdot-}$ at intramembranous sites, and thereby suppresses NADPH-supported lipid peroxidation; this is the main reason for supporting $\text{O}_2^{\cdot-}$ involvement in microsomal lipid peroxidation [5]. Another reason is that lipid peroxidation in microsomes is insensitive to SOD [5,7,24,25], because the enzyme might be inaccessible to $\text{O}_2^{\cdot-}$ generated at the membrane sites [25]. In microsomes solubilized with cholate, where SOD could be easily accessible to $\text{O}_2^{\cdot-}$ -generating sites, SOD (100 units/ml) showed only 30% inhibition of NADPH-supported lipid peroxidation, but MCLA (40 μM) completely inhibited the peroxidation (data not shown). This finding strongly supports the fact that the antioxidant effect of MCLA is not due to scavenging $\text{O}_2^{\cdot-}$ responsible for lipid peroxidation. In addition, a microsomal component distinct from cytochrome P450 directly reduces the iron chelate in the presence of NADPH-cytochrome P450 reductase [11], excluding the possibility that $\text{O}_2^{\cdot-}$ is involved in NADPH-supported lipid peroxidation. Moreover, iron reduction in microsomes occurs anaerobically [26].

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