3-O-Alkylascorbic Acids as Free Radical Quenchers. II. Inhibitory Effects on Some Lipid Peroxidation Models

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We previously found that 3-O-dodecylcarbomethylascorbic acid (3-RASA, 3, HX-0112) exhibited a potent inhibitory effect on biochemical lipid peroxidation and that 3-RASA (3) alleviated myocardial lesions induced by ischemia-reperfusion treatment in rats. In this study we examined the mode of action of 3-RASA (3) on the inhibition of lipid peroxidation. There was no reducing activity by 3-RASA (3) (i.e., no oxide was produced) against ferric ions and superoxide anion radicals. The low reducing activity of 3-RASA (3) against a radical as compared to that of z-tocopherol was obtained by using a stable radical. However, 3-RASA (3) had a potent inhibitory effect, almost equal to that of z-tocopherol, in the model of lipid peroxidation dependent on enzymatic superoxide generation. 3-RASA (3) very strongly inhibited the chain-reaction of the peroxidation induced by Fe2+-linoleic acid hydroperoxide. On the basis of these findings, it appears that the anti-lipid-peroxidative effects of 3-RASA (3) are due to the inhibition of the radical chain-reaction, as a chain-breaking antioxidant.

Keywords 3-O-alkylascorbic acid; 3-O-dodecylcarbomethylascorbic acid; 3-O-decylascorbic acid; HX-0112; HX-0113; lipid peroxidation; antioxidant; linoleic acid hydroperoxide; site specific Fenton reaction; chain-breaking antioxidant

It has recently become clear that active oxygen species (AOS; such as superoxide anion radical, hydroxyl radical, OOH etc.) and free radicals derived from the biochemical utilization of O2 and the prooxidant stimulation of O2 metabolism participate in the development of exacerbation of various kinds of diseases: ischemia-reperfusion disturbances in the brain and heart, rheumatism, inflammation, gastric ulcers, and cancer.1) Although the exact mechanism of lipid peroxidation and the roles of AOS in this biochemical phenomenon are not entirely clear, there have been numerous reports that disturbances of membranes which result in cell damage are derived from AOS-induced lipid peroxidation.2) The average level of lipid peroxidant in plasma is higher in some disease cases than in normal cases3, the lipid peroxidation has been suggested to result from excess superoxide anion radical (superoxide) production.4)

In fact, unsaturated fatty acids, the constituents of cell membranes, are substantially susceptible to oxidation. Additionally, there is considerable evidence that inhibitors of biochemical lipid peroxidation, such as z-tocopherol (Toc.),5) idebenone,6) and cyanidanol7) have demonstrably valuable pharmacological effects. The thiazolizidine derivative, CS-045,8) an ascorbic acid derivative, CV-36119), and ebselen, a selenium compound,10) which were developed as new antioxidants, have been studied for the treatment of angiopathy, postischemia-reperfusion disturbance and inflammation, respectively.

We synthesized a novel series of 3-O-alkylascorbic acids (3-RASA) in order to develop new antioxidants. We found that 3-O-decylascorbic acid (3-RASA (2), HX-0113) and 3-O-dodecylcarbomethylascorbic acid (3-RASA (3), HX-0112) were strong inhibitors of lipid peroxidation in rat liver microsomes.11) HX-0112 and HX-0113, which are stable lipophilic ascorbic acid derivatives, alleviated myocardial lesions induced by ischemia-reperfusion treatment in rats.12) This paper deals with 3-RASA's mode of antioxidative action, using chemical models of lipid peroxidation. We discuss, especially, whether 3-RASA (3) scavenges AOS to participate in biochemical lipid peroxidation or inhibits the radical chain-reaction in linoleic acid peroxidation.

Results

The chemical structures of the various 3-RASA (1–4) investigated are presented in Fig. 1.

Bios proposed a method for assessing antioxidant activity in which such activity was determined by using a stable radical, 2,2-diphenyl-1-picryl hydrazyl (DPPH).13) The reducing activity of 3-RASA, ascorbic acid and Toc. was

![Fig. 1. Chemical Structure of 3-RASA](image)

3-RASA: (1) R = CH3CH2–, (2) R = CH3(CH2)3–, (3) R = CH3(CH2)11COCH2–, (4) R = CH3(CH2)11–.

![Fig. 2. Reducing Activity against DPPH hydrazyl with 3-RASA and Toc.](image)

1, 3-RASA (1); 2, 3-RASA (2); 3, 3-RASA (3); 4, Toc.; 5, ascorbic acid. DPPH 0.1 mm and equimolar amount of compound exist in EtOH. The decrease of absorption of DPPH is continuously monitored by the absorption change at 517 nm.
determined by using DPPH, which is generally available in laboratories in which electron spin resonance experiments are conducted. The stable free radical was reduced by the test compounds, 3-RASA (1–3), ascorbic acid, and Toc (Fig. 2). The reducing activities of 3-RASA (1–3) against this radical was much lower than that of ascorbic acid and Toc.

The redox potential is a valuable index for the electron donating potency required for a biologically beneficial radical scavenger, 3-RASA required much higher potentials than ascorbic acid in electrode oxidation, but the potentials of 3-RASA were estimated to be lower than those of superoxide/hydrogen peroxide\(^{14}\) and those of Fe\(^{3+}/\text{Fe}^{2+}\) (Table I). Our electrochemical results suggest that 3-RASA (3) must be oxidized by superoxide and Fe\(^{3+}\); however, a strong reducing agent has sometimes acted on the prooxidant and stimulated lipid peroxidation. For instance, a transition metal and ascorbic acid at a low concentration synergistically stimulate nonenzymic induced lipid peroxidation.\(^{15}\) The reducing activity of ascorbic acid, Toc, and 3-RASA against Fe\(^{3+}\) was examined. Ascorbic acid and Toc reduced against Fe\(^{3+}\) immediately, but 3-RASA could not reduce against Fe\(^{3+}\) (Table I). However, 3-RASA (1, 2), but not 3-RASA (4) which bears an octadecyl group, demonstrated reducing activity against Fe\(^{3+}\) after the addition of \(\alpha\)-phenanthroline (Table I).

The effects of 3-RASA (1–4) and Toc. on the lipid peroxidation of linoleic acid (LA), which is dependent on enzymatic superoxide generation, were also studied. The addition of ethylenediaminetetraacetic acid (EDTA)–Fe\(^{3+}\) to the xanthine oxidase (XO)-system resulted in the lipid peroxidation of LA, although no activity was observed in the absence of iron (Fig. 3). The effects of 3-RASA (1–4), Toc., superoxide dismutase (SOD), and catalase on the lipid peroxidation of the system are shown in Table II. Our results agreed with Tien’s data\(^{16}\) which showed that the addition of iron as an EDTA-chelate was necessary for XO-dependent peroxidation (XO-peroxidation); and further, indicated that SOD had an almost complete inhibiting effect and that catalase had a partially inhibiting effect. It appeared that 3-RASA (1–4) and Toc., at the molar ratio of 1:100 parts of LA, almost completely inhibited XO-peroxidation. Mannitol, at the molar ratio of about 2:1 parts of LA, resulted in partial inhibition of activity; 3-RASA (2) and Toc., which are more lipophilic compounds than 3-RASA (1), were capable of inhibiting activity at the molar ratio of 1.5 × 10\(^{-5}\) to LA. It is clear, as with the inhibitory effect of SOD, that XO-peroxidation is dependent on the generation of superoxide.

We assessed the effects of 3-RASA (2, 3) on the XO superoxide generation system by continuous monitoring of the reduction against Fe\(^{3+}\)-cytochrome c. 3-RASA (2, 3) did not affect the generating speed and level of superoxide; the ultraviolet (UV) spectra of 3-RASA (2, 3) were not changed under these conditions (data not shown).

The effects of 3-RASA (2, 3) and Toc. on the site-specific induction of lipid peroxidation by iron in charged micelles were studied according to the method of Fukuzawa.\(^{17}\) At pH 3.5 both Fe\(^{3+}\) and \(\text{H}_2\text{O}_2\) were necessary to induce lipid peroxidation in the positively charged tetradecyltrimethylammonium bromide (TTAB) micelles, in spite of the lipophilic acid hydroperoxide (LOOH) which was present in LA. However, at pH 7, peroxidation of LA was induced by the addition of Fe\(^{3+}\) alone (Fig. 4). The Fe\(^{3+}\)-induced

<table>
<thead>
<tr>
<th>Compound</th>
<th>Redox potential(^a) (mV)</th>
<th>Reducing Fe(^{3+}) (%(^b))</th>
<th>FeCl(_3) alone</th>
<th>FeCl(_3)+ (\alpha)-phenanthroline</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-RASA (1)</td>
<td>305</td>
<td>39</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3-RASA (2)</td>
<td>300</td>
<td>32</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3-RASA (3)</td>
<td>380</td>
<td>12</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3-RASA (4)</td>
<td></td>
<td></td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>Toc.</td>
<td></td>
<td></td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td></td>
<td></td>
<td>87</td>
<td>87</td>
</tr>
</tbody>
</table>

\(a\) The redox potentials are determined by differential pulse voltammetry; an Ag/AgCl reference electrode and a glassy carbon working electrode are used. Voltammetric analysis is conducted in 0.01 M phosphate buffer at pH 7.4. \(b\) The compound exists at the molar ratio of 2:1 parts of Fe\(^{3+}\).

**Table II. Inhibitory Effect of Various Scavengers and Antioxidants on XO-Dependent Peroxidation of Dispersed Linoleate**

<table>
<thead>
<tr>
<th>Addition</th>
<th>(\Delta A_{234}) (nm/min)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.029 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>3-RASA (1)</td>
<td>50 µM</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>3-RASA (2)</td>
<td>50 µM</td>
<td>0.022 ± 0.003</td>
</tr>
<tr>
<td>3-RASA (3)</td>
<td>50 µM</td>
<td>0.011 ± 0.002</td>
</tr>
<tr>
<td>3-RASA (4)</td>
<td>50 µM</td>
<td>0.023 ± 0.026</td>
</tr>
<tr>
<td>Toc.</td>
<td>50 µM</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>SOD</td>
<td>50 µg/ml</td>
<td>0.015 ± 0.003</td>
</tr>
<tr>
<td>Mannitol</td>
<td>10 mM</td>
<td>0.024 ± 0.004</td>
</tr>
</tbody>
</table>

The micelle solution of linoleate is made by sodium linoleate (5.7 mM) with XO (6.6 units/ml), Lubrol (1%), \(\text{CH}_3\text{CHO} (35 mM), and EDTA–Fe\(^{3+}\) (0.11 mM EDTA, 0.1 mM FeCl\(_3\)) in 30 mM NaCl, pH 7.5. The rate of diene conjugation is lined up to approximately 4 min, after which a decrease in rate is observed. The rate is determined from the initial velocity of the reaction (mean ± S.D., \(n=5\)).
peroxidation at pH 7 was not observed using Fe\(^{2+}\) and triphenylphosphine (TPP) pretreatment to reduce against LOOH. The peroxidation was partially inhibited by EDTA at 1/2 molar ratio to Fe\(^{2+}\), and it was completely inhibited by TPP at a molar ratio of almost 1:1 to LOOH (Table III). The peroxidation was strongly inhibited by 3-RASA (2, 3) and Toc. at a molar ratio to LA of 1/500 and a molar ratio to LOOH of 1/7.

**Discussion**

We synthesized various novel antioxidants and selected 3-RASA (2, HX-0113) and 3-RASA (3, HX-0112) for further clinical testing. We determined their inhibitory effects of lipid peroxidation in rat liver microsomes and their stability against autooxidation in aqueous solution. In this paper, we attempt to clarify 3-RASA’s mode of action as an antioxidant. This attempt has been a significant addition to understanding the pharmacological effects of the inhibition of lipid peroxidation.

However, the reactive form(s) of AOS involved in superoxide-dependent lipid peroxidation has not been unequivocally established. Certainly there is much evidence of superoxide generation during many biological reactions. Although superoxide itself is relatively unreactive, the hydroxyl radical is a very reactive AOS toward many compounds, including polyunsaturated fatty acids. However, the hydroxyl radical is too short-lived in living organisms and thus it cannot reach the membrane from a distant place in extracellular fluid.

Samuni et al. suggested a site-specific Fenton mechanism in which the binding of a transition metal ion to the biological target is a prerequisite for the production of hydroxyl radical-mediated cell damage. The hydroxyl radical is probably derived from the superoxide as follows:

\[
\begin{align*}
O_2^- + Fe^{3+} & \rightarrow O_2 + Fe^{2+} \\
2O_2^- + 2H^+ & \rightarrow O_2 + H_2O_2 \\
Fe^{3+} + O_2 + H_2O_2 & \rightarrow Fe^{2+} + OH + OH^- 
\end{align*}
\]

Recently, Fukuzawa et al. demonstrated lipid peroxidation induced by a site-specific Fenton mechanism in charged micelles.

On this fairly reasonable hypothesis, we experimented with 3-RASA to find out the reactivity toward superoxide, the hydroxyl radical, and Fe\(^{3+}\). No reactivity of 3-RASA was observed against superoxide and Fe\(^{3+}\), in spite of 3-RASA’s having a redox-potential suitable for oxidation with these entities. Only after the addition of \(\alpha\)-phenanthroline was 3-RASA capable of reducing activity against Fe\(^{3+}\). The reducing ability of 3-RASA toward the Fe\(^{3+}\) \(\alpha\)-phenanthroline complex was inhibited by the steric hindrance of a long alkyl group. The shielding of the hydroxyl group of 3-RASA from Fe\(^{3+}\) is attributable to 3-RASA’s long alkyl chain. The oxidation of 3-RASA with the hydroxyl radical was reported by Cabelli et al.

However, it is too difficult to make an exact comparison of the reactivity of an antioxidant with the hydroxyl radical because of the high reactivity of hydroxyl radical. We chose the stable radical (DPPH), which Blois proposed as an assay for the antioxidant. 3-RASA obviously had less reducing activity against DPPH than did Toc., a typical inhibitor of lipid peroxidation. The low reducing activity and the high redox potential of 3-RASA were in conflict with its potent inhibitory effect on lipid peroxidation. In other words, based on our data, 3-RASA will be characterized as a stable antioxidant.

While many organic compounds which have reducing activity, except for transition metal complexes, are hardly oxidized or only slowly oxidized in aqueous solution by superoxide, 3-RASA was not oxidized in the enzymatic generation of superoxide, although it demonstrated complete inhibition toward lipid peroxidation dependent on superoxide generation. The XO-dependent peroxidation which became necessary with the addition of EDTA-Fe\(^{3+}\) might occur via the hydroxyl radical (see, Eqs 1—3).

The 3-RASA, which bear a long alkyl chain, and Toc. showed potent inhibitory effects, but mannitol and 3-RASA (1), which bear an ethyl group, are lower in activity than the lipophilic hydroxyl-radical scavengers on XO-dependent peroxidation. SOD was also an effective protectant for the oxidation, but catalase did not lead to complete inhibition. The fact that SOD exists in the enzymatic superoxide generation area (outside the micelles) and that it is efficiently able to dismutate superoxide, and the fact that catalase is sensitive to superoxide, account for this phenomenon (see the following section for further details). On the basis of the data, we conclude that 3-RASA is a potent antioxidant. The above observations, while not conclusively demonstrating that 3-RASA is a specific antioxidant by itself, at least indicate that 3-RASA has a potentially stabilizing effect against free radicals.
our results, it is suggested that lipid peroxidation occurring from the site-specific Fenton reaction cannot be effectively inhibited by a hydrophilic antioxidant. In fact, the antioxidant efficiency of 3-RASA was influenced by its hydrophobic action on lipid peroxidation in rat liver microsomes. 11, 17 3-RASA, bearing a long alkyl chain, would be located in the lipid layer and might react as an antioxidant. Our hypothesis is also supported by the fact that the inhibitory effect of 3-RASA (1), which bears an ethyl group, was lower than that of 3-RASA (2), which bears a decyl group, on XO-dependent peroxidation.

It was reported that the antioxidant efficiency of Toc. in liposomes depends on its ability to react mainly with the chain-initiating and chain-propagating lipid radicals. 29, 22 On the basis of these findings, since the mode of antilipid peroxidative action of the lipophilic 3-RASA (2, 3) is thought to be similar to that of Toc., the effects of 3-RASA on the lipid peroxidation model which involved site-specific chain-initiating lipid radicals, not initiated by AOS, were studied. The Fe2⁺−LOOH-initiated peroxidation, which is a minor modification of the lipid peroxidation induced in TTAB micelles by site-specific Fenton reaction, 23 was suitable for our purpose. In this peroxidation, because of the results obtained from TTP treatment and the addition of Fe3⁺, induction is clear, as shown in the following equations:

\[
\text{LOOH} + \text{Fe}^{2+} \rightarrow \text{LO}^- + \text{OH}^- + \text{Fe}^{3+}
\]  

(4)

\[
\text{LO}^- + \text{LH} \rightarrow \text{LOH} + \text{L}^-.
\]  

(5)

The potent inhibitory effects of 3-RASA (2, 3) and Toc. on Fe2⁺−LOOH-induced peroxidation are obviously due to reaction with the lipid radical (s) derived from LOOH. These lipid radicals, like the hydroxyl radical, show very high reactivity. These findings resolved the previous conflict, between the low reducing activity of 3-RASA (2, 3) and the potent inhibitory effects of these compounds on lipid peroxidation models. That is, the conflict probably involves the difference in chemical reactivity between DPPH as a radical and the lipid radicals. Since lipid peroxidation consists of high reactive chain-reactions, the difference in chemical reducing activity between Toc. and 3-RASA against low potential radicals, DPPH, will be compensated for by the high reactivity of lipid radicals, or may be compensated for by the negligible levels of each. Of course, this consideration is based on the localization of 3-RASA, like Toc., 30 in the lipid layer as a site for the radical chain-reaction. Additionally, 3-RASA would be expressed in scavenging activity for same considerably reactive AOS (such as \(\cdot\text{O}_2\)), 31 oxygen and iron complex, 32 but this scavenging activity against remarkably reactive AOS, which is generated only at a distant place from the membrane, may not be very important as a protectant of the biomembranes. On the basis of these findings, it appears that the potent antioxidative effects of 3-RASA (2, 3) on biochemical lipid peroxidation are due to scavenging activity for AOS which participates in the peroxidation, XO-dependent lipid peroxidation, but that 3-RASA (2, 3) inhibits the radical chain-reaction in linoleic acid peroxidation as a chain-breaking antioxidant.