Antioxidative Activity Measurement in Lipid Peroxidation Systems with Malonaldehyde and 4-Hydroxy Nonenal

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Antioxidative activities of vitamin E, ethylenediamine tetraacetic acid (EDTA), ferulic acid, and butylated hydroxy toluene (BHT) were monitored with lipid peroxidation systems. Formation of malonaldehyde (MA) and 4-hydroxy nonenal (4-HN) from ethyl linoleate or rat liver microsome oxidized by $FeCl_2/H_2O_2$ or ADP/$FeSO_4$ was measured by gas chromatography. Over 90% of MA and 4-HN formation was inhibited by 100 mmol/L of vitamin E. A synthetic antioxidant, BHT, showed the strongest antioxidative activity, followed by that of vitamin E, whereas EDTA accelerated formation of MA and 4-HN in the ethyl linoleate/$FeCl_2/H_2O_2$ system. Vitamin E did not suppress lipid peroxidation significantly when microsome was oxidized by ADP/$FeSO_4$. EDTA inhibited oxidation of microsome by ADP/$FeSO_4$ considerably; in contrast, it did not in the case of oxidation by $FeCl_2/H_2O_2$.

KEY WORDS: Antioxidant, lipid peroxidation, malonaldehyde, 4-hydroxy nonenal.

Some naturally occurring antioxidants, such as α-tocopherol, ascorbic acid, and uric acid, reportedly protect biological cells from damage caused by oxidizing agents including singlet oxygen, hydrogen peroxide, superoxide anion, and hydroxy radical (1-3). Among the components most susceptible to an oxidizing agent are lipids. Therefore, biological and chemical oxidation of lipids has been intensively investigated because it is proposed to be associated with aging (4), mutagenesis (5), and carcinogenesis (6). Many researchers have been attempting to find appropriate substances with antioxidative activity to prevent these biological phenomena.

The most commonly used method to monitor oxidation of lipids is the thiobarbituric acid assay (TBA). This method is very useful for measuring the relative degree of lipid oxidation because TBA reacts with many lipid peroxidation products (7). However, it is not possible to monitor a single, specific product by the TBA method. Recently, we developed a new gas chromatographic method to monitor formation of reactive carbonyl compounds, such as malonaldehyde (MA) and 4-hydroxy nonenal (4-HN), from lipids or rat liver microsome (8,9). MA and 4-HN were reacted with N-methylhydrazine (NMH) to yield 1-methylpyrazole and 5-(1-hydroxyhexyl)-1-methylpyrazoline, respectively, which were subsequently analyzed by a gas chromatograph equipped with a high-resolution, fused-silica capillary column and a nitrogen-phosphorus specific detector. In the present study, antioxidative activity of various chemicals in rat liver microsome or ethyl linoleate was measured by a newly developed gas chromatographic method.

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MATERIALS AND METHODS

Materials. Ethyl linoleate and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO). Ferrous chloride, ferrous sulfate, dichloromethane, and chloroform were bought from Fisher Scientific Co., Ltd. (Fair Lawn, NJ). NMH was obtained from Fluka Chemie A.G. (Buchs, Switzerland).

Preparation of rat liver microsome. Liver microsomes were prepared from a male Sprague-Dawley rat (2 mon old, 200 g) according to the method described by Slater and Sawyer (10) with minor modification. The livers (17 g) from two rats were homogenized by a polytron homogenizer, and a 25% homogenate (w/v) was prepared with addition of 0.05 M trizma buffer (pH = 7.4). The microsomes were purified by centrifugation four times and stored at −80°C until used.

Oxidation of microsomes by Fe_2Cl_2/H_2O_2. A trizma buffer solution (3 mL, pH = 7.4) containing 20 mg of protein, 0.6 μmol $FeCl_2$, and 0.3 μmol H_2O_2 was incubated at 37°C for different time periods. The reaction was stopped by the addition of 1.2 mg butylated hydroxy toluene (BHT). MA and 4-HN were determined by the gas chromatographic method (11) and the TBA method (7).

Oxidation of microsomes by ADP/$FeSO_4$/reduced nicotinamide ademine dinucleotide phosphate (NADPH). A trizma buffer solution (3 mL, pH = 7.4) containing 40 mg of protein, 9 μmol NADPH, 6 μmol adenosine diphosphate (ADP), and 0.06 μmol $FeSO_4$ was incubated at 37°C for different time periods. The reaction was stopped by the addition of 2 mg of BHT.

Oxidation of ethyl linoleate by Fe_2Cl_2/H_2O_2. An aqueous solution (5 mL) containing ethyl linoleate (1.5 mg/mL), 0.25 mmol trizma buffer (pH 7.4), 0.75 mmol potassium chloride, and surfactant sodium dodecyl sulfate (0.2%) was stirred with or without 1 μmol $FeCl_2$ and 0.5 μmol H_2O_2 at 37°C for 16 hr. The oxidation reaction was stopped by adding 2 mg of BHT.

Oxidation of microsomes and ethyl linoleate in the presence of antioxidants. Vitamin E (1-100 μmol/L), ethylenediamine tetraacetic acid (EDTA, 100 μmol/L), ferulic acid (100 μmol/L), and BHT (100 μmol/L) were added to the above microsome or ethyl linoleate samples before addition of Fe_2Cl_2 or $FeSO_4$ and then the samples were treated as in the procedures described above.

MA and 4-HN formed in the samples were analyzed by the gas chromatographic method (11). The oxidized samples were also subjected to the TBA method (7).

RESULTS AND DISCUSSION

The amount of MA formed from microsomes upon $FeCl_2/H_2O_2$ or ADP/$FeSO_4$ oxidation increased over incubation time, reached a maximum around 40 min, and then plateaued. Therefore, a 40-min incubation time was used for the further experiments on microsomes. Table 1 shows the amount of MA formed from ethyl linoleate and microsomes with or without oxidants. The values from the TBA assay are calculated as MA. Certain amounts

TABLE 1

Amount of MA Formed from Ethyl Linoleate and Rat Liver Microsome Oxidized by $FeCl_2/H_2O_2$ or ADP/$FeSO_4$

Sample	Incubation time	Oxidation agent	Measured by[a] GC	TBA
Ethyl linoleate	0	[b]	0.6 ± 0.2	0.6 ± 0.2
	16 hr	[b]	0.8 ± 0.1	0.9 ± 0.5
	16 hr	$FeCl_2/H_2O_2$	19.3 ± 5.7	21.5 ± 4.0
Microsome	0	[b]	2.2 ± 0.6	1.4 ± 0.9
	40 min	[b]	1.3 ± 0.4	0.9 ± 0.1
	40 min	$FeCl_2/H_2O_2$	11.1 ± 0.5	14.8 ± 1.7
	40 min	ADP/$FeSO_4$	43.7 ± 7.8	59.7 ± 5.2

[a]Values are mean (nmol/mg fatty acid or protein) ± standard deviation (n = 3).
[b]Oxidation agent was not used.

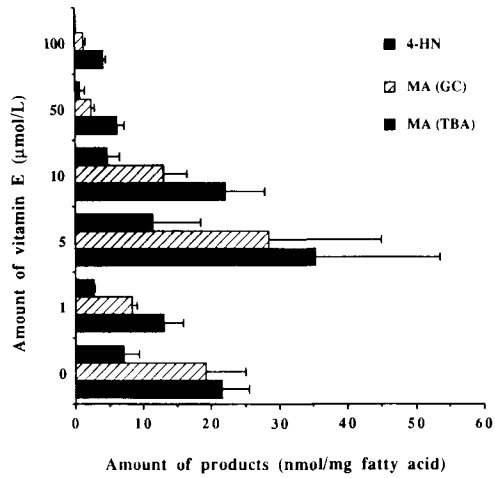

FIG. 1. Effect of vitamin E on the production of MA and 4-HN from ethyl linoleate oxidized by $H_2O_2/FeCL_2$ for 16 hr. Values are mean ± standard deviation (n = 3).

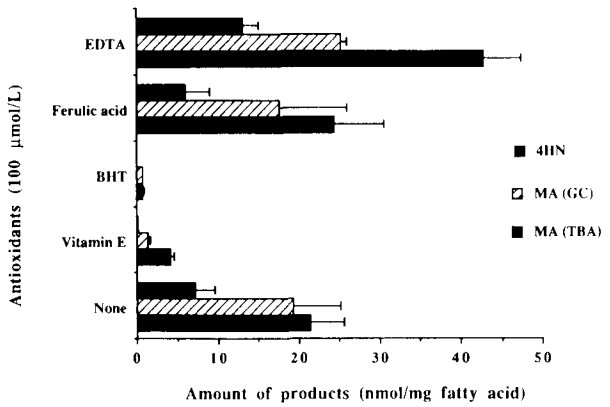

FIG. 2. Effect of antioxidants on the product of MA and 4-HN from ethyl linoleate oxidized by $H_2O_2/FeCl_2$ for 16 hr. Values are mean ± standard deviation (n = 3).

(0.6–4.0 nmol/mg) of MA were already present in the blank samples, which is consistent with our previous report (9).

4-HN was detected (7.1 ± 2.4 nmol/mg) only in the ethyl linoleate sample oxidized by $FeCl_2/H_2O_2$.

Generally, the TBA method gives overestimated values for MA (8), but the results in the present study showed only slight differences between the values from TBA and gas chromatographic (GC) methods. This may be due to the difference between *in vivo* and *in vitro* methods. These preliminary experiments have proven that these lipid peroxidation model systems can be used to examine the antioxidative activity of chemicals.

Figure 1 shows the antioxidative activities of vitamin E measured in an ethyl linoleate system. It is interesting that the addition of 5 μmol/L of vitamin E increased oxidation and that antioxidative activity appeared when the dose was increased to over 50 μmol/L. Over 90% of the formation of MA (GC) and 4-HN was inhibited by 100 μmol/L of vitamin E. Therefore, antioxidative activities of other chemicals were measured at the dose of 100 μmol/L to compare their activity with that of vitamin E.

Figures 2 and 3 show the antioxidative activities of EDTA, ferulic acid, BHT, and vitamin E measured in an ethyl

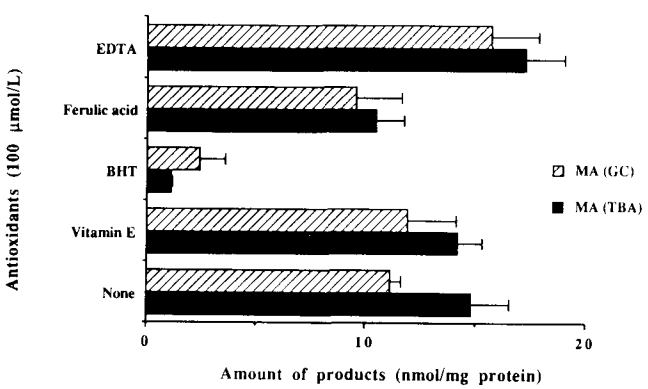

FIG. 3. Effect of antioxidants on the production of MA and 4-HN from a rat liver microsome oxidized by $H_2O_2/FeCl_2$ for 40 min. Values are mean ± standard deviation (n = 3).

linoleate system and a microsome system oxidized by $FeCl_2/H_2O_2$. It is obvious that BHT, a synthetic antioxidant, showed the strongest antioxidative activity, followed by vitamin E. Addition of EDTA accelerated formation of MA and 4-HN, suggesting that lipid peroxidation was induced by an almost equivalent amount of iron (II) chelate (12).

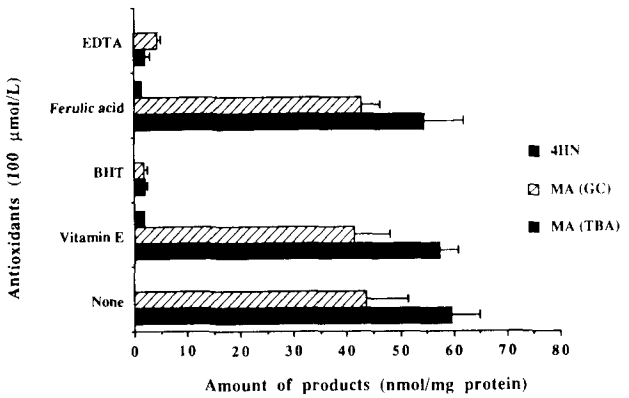

FIG. 4. Effect of antioxidants on the production of MA and 4-HN from a rat liver microsome oxidized by ADP/$FeSO_4$ for 40 min. Values are mean ± standard deviation (n = 3).

Figure 4 shows the antioxidative activity of EDTA, ferulic acid, BHT, and vitamin E measured in a microsome system oxidized by ADP/$FeSO_4$. Vitamin E did not suppress lipid peroxidation significantly, but formation of 4-HN was observed. In contrast to the systems oxidized by $FeCl_2/H_2O_2$, EDTA inhibited oxidation considerably in the systems oxidized by ADP/$FeSO_4$. Trapping ferrous ion by EDTA seems to be more effective in the ADP/$FeSO_4$ system than in the $FeCl_2/H_2O_2$ system. Tien *et al.* (13) reported that the rate of Fe^{2+} autoxidation in microsomal lipid peroxidation with NADPH is strictly a function of the EDTA/Fe^{2+} ratio. EDTA/Fe^{2+} ratios close to 1:1 promote lipid peroxidation, whereas EDTA/Fe^{2+} ratios above 1:1 inhibit lipid peroxidation (14). The EDTA/Fe^{2+} ratio used in the present study was 1:2, suggesting that no inhibition would occur.

The hydrophilic surface of the microsome membrane may inhibit vitamin E from contacting the fatty acid moiety of microsome (15). On the other hand, vitamin E directly contacts ethyl linoleate and scavenges hydroxy radicals, resulting in the occurrence of antioxidative activity.

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