Antioxidative Behaviors of 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone and 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone against Lipid Peroxidation

Takuro Koga,*,[†] Keiko Moro,[†] and Takanao Matsudo[‡]

Noda Institute for Scientific Research, 399 Noda, Noda-shi, Chiba 278, Japan, and Research and Development Division, Kikkoman Corporation, 399 Noda, Noda-shi, Chiba 278, Japan

Natural flavor compounds, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF), were evaluated for antioxidative behaviors against lipid peroxidations. They inhibited hydroperoxidation of methyl linoleate initiated by a lipid-soluble azo compound, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), in solution. The antioxidative activities of HDMF and HEMF were less than that of ascorbic acid when the emulsified methyl linoleate oxidized by a water-soluble azo compound, 2,2′-azobis(2-amidinopropane) dihydrochloride, while they were more effective than ascorbic acid when lipid peroxidation was initiated by a lipid-soluble AMVN. These furanones were also more effective than ascorbic acid in the inhibition of the formation of cholesteryl ester hydroperoxides in plasma. HEMF suppressed the oxidation of low-density lipoprotein without any synergistic effect with α-tocopherol. In the autoxidation of rat brain homogenate, HDMF and HEMF acted as inhibitors, while ascorbic acid acted as a prooxidant. These results indicate that HDMF and HEMF are potent antioxidants so that they would be important components not only in exhibiting desirable flavor but also in inhibiting oxidative deterioration in foods.

INTRODUCTION

4-Hydroxy-3(2*H*)-furanone derivatives such as 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF) are known natural flavor components (Figure 1). HDMF is found in fruits (Rodin et al., 1965; Mayerl et al., 1989) and heat-processed foods (Tonsbeek et al., 1968; Takei and Yamashita, 1974; Tressl et al., 1978; Takei, 1977; Schieberle, 1990, 1991). HEMF was first identified as a character impact factor of Japanese-style soy sauce (shoyu) (Nunomura et al., 1976) and was also found in fermented soybean paste (miso) (Sugawara et al., 1994). The biosynthesis of HDMF and HEMF by microorganism from sugar phosphate has also been reported (Sasaki et al., 1991; Hecquet, 1996). Synthetic HDMF is largely used as an additive exhibiting caramel-like odor in the food industry.

It has been reported that HDMF and HEMF are effective in inhibiting benzo[*a*]pyrene-induced mouse forestomach neoplasia when fed in the diet (Benjamin et al., 1991; Nagahara et al., 1992; Kataoka et al., 1997). These authors also found that both furanones acted as reductants and were antioxidants by inhibiting hydroxy radical-mediated lipid peroxidation and by scavenging singlet oxygen (Nagahara et al., 1992; Kataoka et al., 1997). However, little is known about the antioxidative
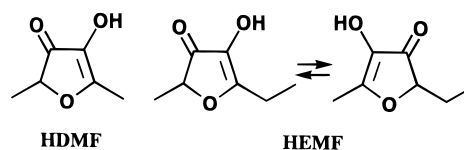

Figure 1. Chemical structures of HDMF and HEMF.

activity of these furanones in lipid peroxidations mediated by peroxyl radical and occurring in biological systems.

In this work, we examined the activity of HDMF and HEMF as antioxidants by the inhibition of peroxyl radical-driven hydroperoxidation of methyl linoleate in solution and in an aqueous dispersion. We also examined the antioxidant status of HDMF and HEMF in biological constituents such as plasma, low-density lipoprotein (LDL), and brain homogenate and compared them with those of other well-known antioxidants such as ascorbic acid, which is also a furanone analogue.

MATERIALS AND METHODS

Chemicals. HDMF, HEMF, methyl linoleate, and methyl laurate were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Methyl linoleate was purified to remove contaminant hydroperoxides by column chromatography (Terao and Matsushita, 1986). D-α-Tocopherol was obtained from Eisai Co. (Tokyo, Japan). L-Ascorbic acid was purchased from Sigma Chemical Co. (St. Louis, MO). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), butylated hydroxytoluene [2,6-di-*tert*-butyl-4-methylphenol; BHT], and Triton X-100 were purchased from Wako Pure Chemical Co. (Osaka, Japan). All other chemicals and solvents were of analytical grade.

* Author to whom correspondence should be addressed (telephone 81-471-23-5569; fax 81-471-23-5550; e-mail tkoga@mail.kikkoman.co.jp).
[†] Noda Institute for Scientific Research.
[‡] Kikkoman Corp.

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Preparation of Human Blood Plasma and LDL. Blood was taken from healthy volunteers and then was put into tubes containing ethylenediaminetetraacetic acid disodium salt (1 mg/mL). Plasma was separated by centrifugation at 1000*g* for 20 min at 4 °C and was stored at −80 °C until use, but for a period not exceeding 1 week. LDL was isolated from fresh plasma in the density range 1.019−1.063 g/mL by ultracentrifugation as previously described (Goldstein et al., 1983). Concentration of protein was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Peroxyl Radical-Scavenging Activity in Solution. Peroxyl radical-scavenging activities of HDMF and HEMF in homogeneous solution were determined by measuring the inhibition of free radical oxidation of methyl linoleate that was initiated by a lipid-soluble azo compound, AMVN (Niki et al., 1984). The reaction mixture contained 330 μmol of methyl linoleate and 0.11 μmol of antioxidant in 1.0 mL of *tert*-butyl alcohol/methanol (3:1, v/v). Oxidation was initiated by adding 110 mM AMVN (100 μL) in the same solvent. The final mixture was incubated at 37 °C with continuous shaking in the dark, and aliquots were withdrawn at regular intervals to measure the formation of methyl linoleate hydroperoxides (MeL-OOH).

Oxidation of Emulsified Methyl Linoleate. The inhibitory effects of HDMF and HEMF in the oxidation of emulsified methyl linoleate were measured (Yamamoto et al., 1984). Methyl linoleate was placed in a test tube and dispersed with a vortex mixer for 1 min in 1 mL of Triton buffer, that is, Tris-HCl buffer (0.01 mM, pH 7.4) with or without antioxidants containing 0.1% Triton X-100 as emulsifier, followed by ultrasonic irradiation with an ultrasonic liquid processor (model XL2020, Misonix Inc., New York) for 30 s. In the experiment with a water-soluble azo compound, AAPH dissolved in Triton buffer was added to the mixture and shaken vigorously on a vortex mixer for 1 min. In the experiments with a lipid-soluble azo compound, AMVN dissolved in methyl laurate was dispersed in Triton buffer with a vortex mixer for 1 min followed by ultrasonic irradiation for 30 s, and then the AMVN mixture was added to the reaction mixture followed by vigorous shaking on a vortex mixer for 1 min. In both experiments, oxidation was carried out in the dark at 37 °C under air with continuous shaking. After 3 h of incubation, the reaction mixture (100 μL) was withdrawn and was mixed with methanol (200 μL) to measure the formation of MeL-OOH.

Measurement of MeL-OOH. The formation of MeL-OOH was measured according to the method of Terao and Matsushia (1986) using high-performance liquid chromatography (HPLC) with UV detection at 235 nm. Samples were injected into a silica column (Inertsil SIL, 6 × 150 mm, 5 mm; GL Science, Tokyo, Japan) and eluted with hexane/2-propanol (99:1, v/v) at a flow rate of 2.5 mL/min.

Oxidation of Plasma and LDL. Two milliliters of plasma was mixed with 200 μL of phosphate-buffered saline (PBS, pH 7.4) containing 1.2 mM antioxidant and 6 mM diethylenetriaminepentaacetic acid for preventing the prooxidant effect of contaminant metal ion in a test tube. The oxidation was initiated by the addition of 200 μL of AAPH (120 mM) dissolved in PBS or 40 μL of AMVN (600 mM) dissolved in ethanol and 160 μL of PBS. The final mixture was incubated at 37 °C in the dark under air with continuous shaking. A portion of the reaction mixture (200 μL) was withdrawn after incubation for 4 and 17 h in the AAPH- and the AMVN-induced oxidation, respectively. In the oxidation of LDL, 1 mL of LDL (1 mg of protein/mL) was mixed with 900 μL of PBS containing antioxidant. The oxidation of LDL suspension was initiated by the addition of 100 μL of 100 μM CuSO$_4$ dissolved in PBS and was carried out at 37 °C in the dark under air with continuous shaking. At specific time intervals, an aliquot of the suspension (200 μL) was withdrawn. In both cases, 3 mL of 2.5 mM BHT in methanol was added to a sample of the reaction mixture, and the mixture was sonicated in an ultrasonicator (Branson, model B1210J-DTH, Yamato Co., Tokyo, Japan) for 1 min. *n*-Hexane (3 mL) was added to the mixture followed

by centrifugation at 1500*g* for 3 min. Hexane layers were collected after three extractions and evaporated under a stream of nitrogen gas. The residue was dissolved in a mixture of acetonitrile and chloroform (3:2, v/v, 100 μL) for HPLC analyses of cholesteryl ester hydroperoxides (ChE-OOH) and α-tocopherol.

Measurement of ChE-OOH. ChE-OOH was measured by HPLC on an octyl-bonded silica column (TSK gel OCTYL-80 Ts column, 4.6 × 100 mm; TOSOH, Tokyo Japan) using acetonitrile/chloroform (92.5:7.5, v/v) as an eluent at a flow rate of 1.0 mL/min. ChE-OOH were detected by ultraviolet absorption at 235 nm, and their concentrations were calculated from the standard curve of the hydroperoxy derivative of cholesteryl linoleate (Arai et al., 1997).

Measurement of α-Tocopherol. α-Tocopherol was measured by HPLC on an octyl-bonded silica column (TSK gel OCTYL 80Ts, 4.6 × 100 mm, TOSOH) using acetonitrile/water (99:1, v/v) as an eluent at a flow rate of 1.2 mL/min. The eluent was monitored fluorometrically at an excitation wavelength of 298 nm and an emission wavelength of 325 nm using a Shimadzu RF-10A (Shimadzu Co., Kyoto, Japan). The concentration was calculated from the standard curve of the authentic compound.

Assay of Autoxidation of Rat Brain Homogenate. The supernatant fraction of rat brain homogenate was prepared according to the method of Stocks et al. (1974) with slight modification. In brief, 6-week-old male Wistar rats (Charles River Japan Inc., Atsugi, Japan) were used. After decapitation, whole brains were removed, washed with PBS, and weighed. Five grams of brain was minced and homogenized by using a Polytron homogenizer PT10SK (Kiematica, Switzerland) in 30 mL of cold 0.1 M Tris-HCl buffer (pH 7.4) containing 0.135 M KCl. The homogenate was centrifuged at 1500g for 10 min at 4 °C. A 1 mL sample of supernatant was removed and diluted with 4 mL of 0.1 M Tris-HCl buffer (pH 7.4) containing 0.135 M KCl and antioxidants. The sample mixture was incubated at 37 °C in the dark under air with continuous shaking. At specific time intervals, an aliquot of the reaction mixture (100 μL) was withdrawn, and the thiobarbituric acid (TBA) assay was carried out according to the method of Uchiyama and Mihara (1978). The amount of TBA reactive substances (TBARS) was expressed as micromolar malondialdehyde. Tetraethoxypropane was used as the standard compound.

RESULTS

Peroxyl Radical-Scavenging Activity of HDMF and HEMF in Solution. Peroxyl radical-driven peroxidation of methyl linoleate was carried out in a solution of *tert*-butyl alcohol and methanol using a lipophilic radical generator, AMVN. MeL-OOH, the primary products of peroxidation, accumulated during the incubation in the absence of antioxidant (Figure 2). α-Tocopherol gave an obvious induction period followed by accelerated hydroperoxidation. Each furanone also suppressed the oxidation of methyl linoleate, although the reaction curve showed no distinct induction period. The inhibitory effects in the presence of HDMF and HEMF were indistinguishable from one another in this solution system and were lower than that in the presence of ascorbic acid.

Inhibition of Free Radical-Initiated Peroxidation of Emulsified Methyl Linoleate by HDMF and HEMF. The emulsified methyl linoleate was oxidized by water-soluble and lipid-soluble azo compounds. The rates of initiation (R_i) were 1.89 × 10^{-8} and 7.41 × 10^{-8} M/s, which were caluculated from the induction period produced in the presence of trolox and α-tocopherol in the oxidation induced by AAPH and AMVN, respectively (Barclay et al., 1984). The effects of HDMF and HEMF on this reaction system were measured at a concentra-
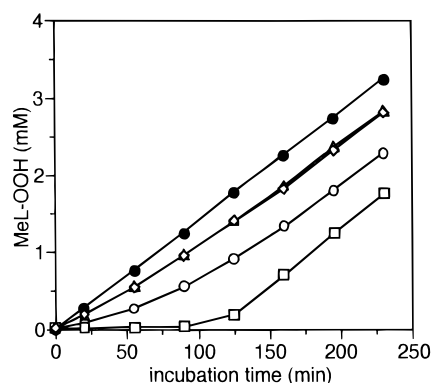
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Figure 2. Inhibition of oxidation of methyl linoleate by α-tocopherol, ascorbic acid, HDMF, and HEMF induced by AMVN in solution at 37 °C in the dark under air: (●), no addition; (□), α-tocopherol; (○), ascorbic acid; (△), HDMF; (◇), HEMF. The reaction system consisted of methyl linoleate (300 mM), antioxidant (100 μM), and AMVN (10 mM) in *tert*-butyl alcohol/methanol (3:1, v/v). The data are typical of several separate experiments.
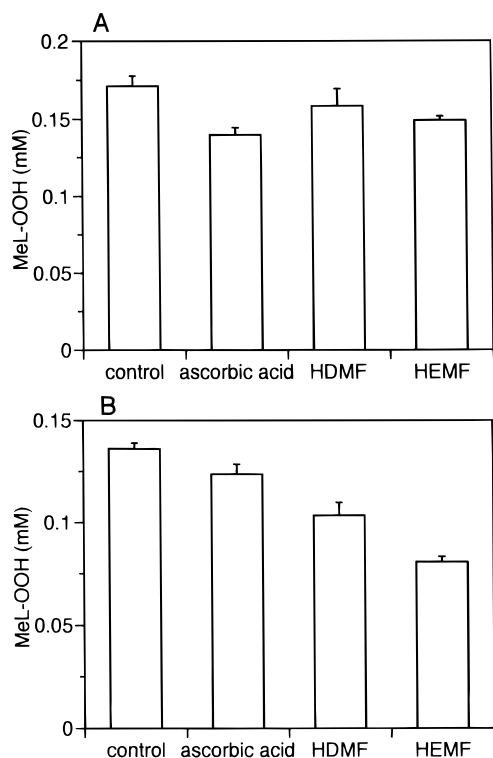

Figure 3. Inhibition of oxidation of emulsified methyl linoleate by ascorbic acid, HDMF, and HEMF induced by AAPH (A) and AMVN (B) at 37 °C in the dark under air: (A) reaction system consisted of 100 mM methyl linoleate, 100 μM antioxidant, 20 mM AAPH, and 0.1% Triton X-100; (B) reaction system consisted of 47.6 mM methyl linoleate, 476 μM antioxidant, 9.5 mM AMVN, 0.1% Triton X-100, and 10% methyl laurate (v/v). The mixture was incubated for 3 h. Bars represent the mean ± the standard deviation of three separate experiments.

tion of 100 μM (Figure 3) and were compared with that of ascorbic acid after 3 h of oxidation. When the oxidation was initiated by water-soluble AAPH, the antioxidative activities of HEMF and HDMF were weaker than that of ascorbic acid. On the other hand, when the oxidation was initiated by lipid-soluble AMVN, HDMF and HEMF suppressed the formation of MeL-OOH more efficiently than ascorbic acid. It should be
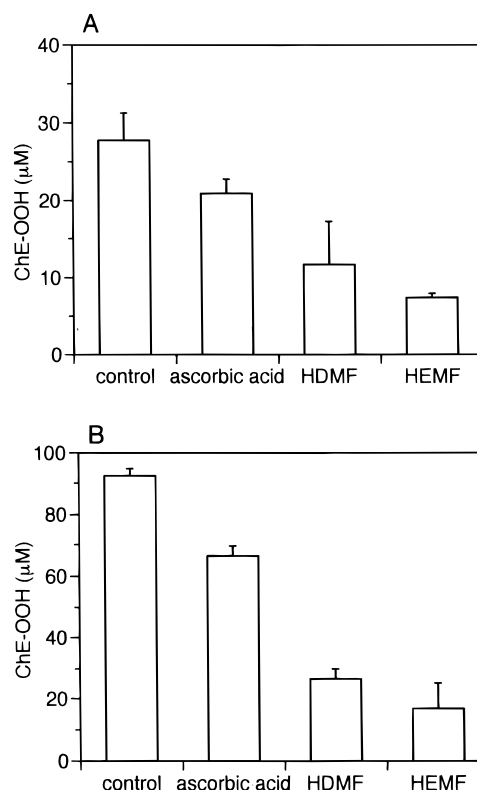

Figure 4. Inhibition of oxidation of human plasma by ascorbic acid, HDMF, and HEMF induced by AAPH (A) and AMVN (B) at 37 °C in the dark under air. The reaction system consisted of antioxidant (200 mM), diethylenetriaminepentaacetic acid (0.5 mM), and AAPH (30 mM) or AMVN (10 mM) in a suspension of plasma (83.3%, v/v) in PBS. The final mixture was incubated for 5 (A) and 17 h (B). Bars represent the mean ± the standard deviation of three separate experiments. The initial concentrations of endogenous α-tocopherol, ascorbic acid, and uric acid in the reaction mixture were 26.0, 101.3, and 244.4 μM, respectively.

noted that HEMF was much more effective than HDMF in inhibiting the AMVN-induced oxidation of emulsified methyl linoleate, although a little difference was observed between the two compounds in the AAPH-induced oxidation.

Inhibition of Free Radical-Initiated Peroxidation of Human Plasma by HDMF and HEMF. Figure 4 shows the hydroperoxide formation in plasma exposed to azo compounds. Endogenous α-tocopherol, ascorbic acid, and uric acid were found in plasma at 31.2, 121.6, and 293.3 μM levels, respectively. When HDMF, HEMF, or ascorbic acid was added to the reaction mixture at a concentration of 100 μM, the formation of ChE-OOH was suppressed as compared to results in the absence of any antioxidant in the system. Ascorbic acid was less effective than two hydroxyfuranone derivatives in plasma exposed to AAPH and AMVN. The addition of HEMF resulted in the largest inhibition of formation of ChE-OOH in plasma.

Inhibition of Cupric Ion-Induced Oxidation of LDL by HEMF. The effect of HEMF on the oxidation of LDL has also been studied. Figure 5 shows the formation of ChE-OOH and the consumption of α-tocopherol in the oxidation of LDL induced by cupric sulfate in the presence of ascorbic acid and HEMF. When HEMF was added in the oxidation of LDL, the formation of ChE-OOH was suppressed without any apparent induction period and α-tocopherol was consumed at the
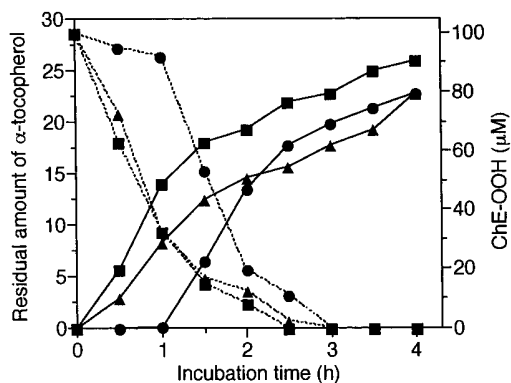
Figure 5. Effects of ascorbic acid and HEMF on the rates of formation of ChE-OOH (solid line) and of consumption of α-tocopherol (dotted line) during the oxidation of human LDL induced by cupric sulfate at 37 °C in the dark under air: (■), no addition; (●), ascorbic acid; (▲), HEMF. The reaction system consisted of LDL (0.5 mg of protein/mL), cupric sulfate (5 μM), and antioxidant (10 μM) in PBS. The initial concentration of endogenous α-tocopherol in the reaction mixture was 4.1 μM. The data are typical of several separate experiments.
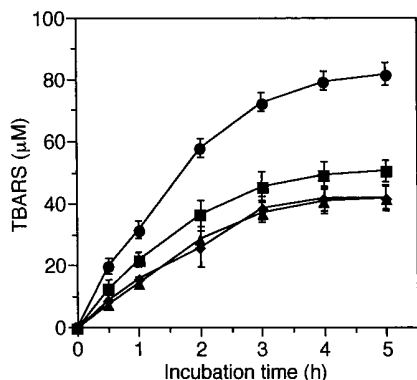

Figure 6. Effects of ascorbic acid, HDMF, and HEMF on the autoxidation of rat brain homogenate at 37 °C in the dark under air: (■), no addition; (●), ascorbic acid; (◆), HDMF; (▲), HEMF. Rat brain homogenate was incubated in the absence and presence of antioxidant (100 μM). Each point represents the mean ± the standard deviation of three separate experiments.

same rate as in the absence of inhibitor. Similar results were obtained when HDMF was added in the oxidation of LDL (data not shown). On the other hand, ascorbic acid suppressed the oxidation of LDL with a clear induction period and reduced the rate of α-tocopherol consumption during induction period.

Inhibition of the Autoxidation of Rat Brain Homogenate by HDMF and HEMF. The formation of TBARS on the time course of autoxidation of rat brain homogenate is illustrated in Figure 6. The effects of HDMF, HEMF, and ascorbic acid on this reaction system were measured at a concentration of 50 μM. The formation of TBARS was suppressed by the addition of HDMF and HEMF, and there was no significant difference between them. The oxidation was accelerated when ascorbic acid was added to rat brain homogenate.

DISCUSSION

The above results show that HDMF and HEMF act as antioxidants by scavenging peroxyl radicals. We observed the disappearance of peroxyl radical-scavenging ability of HDMF and HEMF in their derivatives binding glucose to the 4-OH position (S. Kitao, T.

Matsudo, T. Koga, T. Horiuchi, H. Sekine, unpublished results). This result and an aspect of the chemical structures of HDMF and HEMF (Figure 1) lead to the idea that their antioxidant properties can be attributed to the hydroxy group at the 4-position, which easily donates the hydrogen atom to a peroxyl radical to suppress peroxidation as indicated in conventional chain-breaking antioxidants such as tocopherols and in furanone analogues including ascorbic acid. The lack of a distinct induction period in the inhibition of free radical-induced peroxidation of methyl linoleate in solution by HDMF and HEMF (Figure 2) can be explained by the idea that the rate of their antioxidant activity is similar to the rate of chain propagation of methyl linoleate hydroperoxidation. When the emulsified methyl linoleate or plasma was oxidized by AAPH and AMVN, HDMF and HEMF also gave no distinct induction periods. Since the patterns of oxidation in the presence of antioxidant were similar relative to the antioxidant efficiency for tested antioxidants in various concentrations in the oxidation of emulsified methyl linoleate and plasma induced by various concentrations of both types of initiators (data not shown), their inhibitory effects were compared after a set period of the oxidation (Figures 3 and 4).

The antioxidative activities of HDMF and HEMF were lower in the emulsion exposed to water-soluble AAPH than that of ascorbic acid (Figure 3). However, the opposite trend was observed when lipid-soluble AMVN was used. HDMF and HEMF were more effective in the AMVN-induced oxidation of emulsified methyl linoleate. This difference seems to be due to their physical properties. The antioxidative activity in a heterogeneous phase such as an emulsion is known to be determined not only by chemical reactivities but also by physical factors (Huang et al., 1997). For example, hydrophobicity and solubility of antioxidants affect their concentrations in different locations in heterogeneous systems. Additionally, the antioxidative activity is affected by their diffusion rates, stability, and degree of dissociation, which may change with their location in these systems. HDMF and HEMF are soluble in both water and organic phase, so they would scavenge the peroxyl radicals from both AMVN and lipids generated in lipid phase in emulsion, which are not accessible to ascorbic acid, which is slightly soluble in an apolar solvent (Niki et al., 1984). In contrast with AMVN-induced oxidation of emulsified methyl linoleate, when the oxidation was initiated by AAPH, water-soluble ascorbic acid may scavenge aqueous radicals generated from AAPH in a water phase more efficiently than HDMF and HEMF, resulting in a superior inhibition.

HDMF and HEMF are homologues of 4-hydroxy-3(2*H*)-furanone with methyl and ethyl groups at the 2(or 5)- and 5(or 2)- positions, respectively. Although their structures are identical except for the substituents at the 2(or 5)-position and their peroxyl radical-scavenging abilities were indistinguishable from one another in homogeneous solution, the antioxidative activity of HEMF was higher than that of HDMF in emulsion. Kataoka et al. (1997) also reported that HEMF is more potent than HDMF in the inhibition of hydroxy radical-mediated peroxidation of emulsified linoleic acid. This difference may originate from their different hydrophobicities. HEMF having ethyl and methyl groups is rather lipophilic as compared to HDMF having two

methyl groups and seems to be more accessible than HDMF to the lipid phase where the lipid peroxidation takes place.

It is noteworthy that both furanones were more effective than ascorbic acid, which is the most effective aqueous-phase antioxidant in plasma (Frei et al., 1989), in the inhibition of peroxidation of plasma exposed to azo compounds. Probably, the effective concentration of antioxidant and its mobility at the microenvironment would be important factors in the inhibition of oxidation of plasma. It may be possible that lipophilic HDMF and HEMF are located within the lipids in the lipoproteins and those bound to transfer protein which are the target of lipid peroxidation in plasma (Frei et al., 1988). In fact, HEMF was effective in the inhibition of LDL oxidation (Figure 5).

It has previously been found that ascorbic acid can regenerate α-tocopherol by reacting with α-tocopheroxy radical (Niki et al., 1984; Doba et al., 1985). HEMF suppressed the oxidation of LDL but did not reduce the rate of α-tocopherol consumption (Figure 5). There seems to be little interaction of HEMF with α-tocopherol such as a repair of α-tocopherol radicals. HEMF seems to inhibit the oxidation of LDL independent of α-tocopherol.

It is known that the action of an antioxidant may switch from anti- to prooxidant, depending on its concentration and the presence of transition metal ions (Terao and Matsushita, 1986; Yamamoto et al., 1987). No prooxidant effect of HDMF and HEMF was observed in the autoxidation of rat brain homogenate, while ascorbic acid accelerated the formation of TBARS. Transition metals presented in biological material are known to account for the autoxidation of brain homogenate (Stocks et al., 1974). Ascorbic acid is a strong reducing agent and may function as a prooxidant in the presence of metal ions (Yamamoto et al., 1987). 4-Hydroxy-3(2*H*)-furanones seem to be less effective as reducing agents for transition metal ions in biological fluids, although HEMF and HDMF are more potent than ascorbic acid in the reduction of bathophenanthroline in the presence of such iron (Nagahara et al., 1992; Kataoka et al., 1997).

In conclusion, HDMF and HEMF act as both water-soluble and lipid-soluble antioxidants by scavenging peroxyl radicals. The structure, 4-hydroxy-3(2*H*)-furanone, can be useful as a key structure to design a potent antioxidant. It is possible that the antioxidative activity of these furanones contributes to their anticarcinogenic effect as proposed previously (Nagahara et al., 1992; Pariza, 1994; Kataoka et al., 1997). HDMF and HEMF would play an important role not only in imparting a desirable flavor in foods but also in increasing the oxidative stability of the systems.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); BHT, butylated hydroxytoluene; ChE-OOH, cholesteryl ester hydroperoxides; HDMF, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone; HEMF, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; MeL-OOH, methyl linoleate hydroperoxides; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid reacting substances

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