Formation and Inhibition of Genotoxic Glyoxal and Malonaldehyde from Phospholipids and Fish Liver Oil upon Lipid Peroxidation

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Many aldehydes formed from lipid peroxidation are reportedly implicated in various diseases, including arteriosclerosis. Lecithin, a blood plasma lipid, and cod liver oil (known to have a beneficial effect on coronary artery disease) were oxidized by Fenton's reagent. The oxidation products, malonaldehyde (MA) and glyoxal, were analyzed in the presence of α-tocopherol or 2″-*O*-glycosylisovitexin (2″-O-GIV) by gas chromatography after derivatization to 1-methylpyrazol and quinoxaline, respectively. α-Tocopherol inhibited MA and glyoxal formation effectively from 5 mg of lecithin at low doses (1 μmol), whereas 2″-O-GIV required 6 μmol to produce the same level of inhibition. However, α-tocopherol did not exhibit a satisfactory effect in the case of cod liver oil compared with 2″-O-GIV. Moreover, addition of α-tocopherol to cod liver oil increased glyoxal formation slightly. The results of the present study suggested that 2″-O-GIV is an excellent antioxidant for fish oils.

Keywords: *Cod liver oil; glyoxal; malonaldehyde; 2″-O-glycosylisovitexin; phospholipids*

INTRODUCTION

A strong relationship between arteriosclerosis and amounts of lipid peroxidation products in the inside wall of arteries was suggested in the early 1950s (Glavind et al., 1952). Later, the progress of arteriosclerosis was found to correspond to increase in the thiobarbituric acid (TBA) values of an artery wall (Aoyama et al., 1965; Iwakami et al., 1965). It was hypothesized that lipid peroxidation products interacted with proteins in the artery wall, and the resulting complex products caused accumulation of cholesterol or cholesterol esters on the same location (Fukuzumi and Iwata, 1963; Fukuzumi, 1965). Among lipid peroxidation products, reactive carbonyl compounds such as malonaldehyde (MA), glyoxal, acrolein, and formaldehyde directly cross-link to proteins and bind covalently to nucleic acids (Lam et al., 1986) and consequently cause biological complications including carcinogenesis (Nair et al., 1986; Furihata et al., 1989). Therefore, these reactive carbonyl compounds can possibly play an important role in arteriosclerosis associated with lipid peroxidation.

A variety of studies have shown an association between increased fish consumption and a decreased rate of coronary artery disease (Simpoulos et al., 1986). These clinical findings may derive from a number of specific effects attributed to $\omega-3$ polyunsaturated fatty acids, such as an inhibition of blood clotting and a lowering of serum cholesterol levels and blood pressure. Although fish oil supplementation reportedly had a beneficial effect on blood pressure and serum triacylglycerols in patients treated for coronary artery disease (Bairati et al., 1992), one should consider that many reactive carbonyl compounds would form from its polyunsaturated fatty acids (Niyati-Shirkhodaee and Shibamoto, 1992) when it is used as a therapeutic tool for certain diseases. Massive doses of fish oil may cause some biological complications. For example, Igarashi and Miyazawa (1985) reported that the TBA value in liver and kidney from a rat fed cod liver oil was significantly greater than that from a rat fed corn oil. The major polyunsaturated fatty acids in fish oils are eicosapentaenoic acid (EPA, $C_{20:5n-3}$, approximately 10%) and docosahexaenoic acid (DHA, $C_{20:6n-3}$ 30–33%) (Kinsella, 1987), while corn oil contains mainly linoleic acid ($C_{18:2n-6}$) and linolenic acid ($C_{18:3n-3}$). These results suggest that cod liver oil produced more TBA-reactive compounds, including MA and glyoxal, than did corn oil *in vivo*. On the other hand, $n-3$ fatty acids such as DHA, EPA, and linolenic acid were more stable than $n-6$ fatty acids (linoleic and arachidonic) against oxidation in an aqueous solution (Miyashita et al., 1993).

Reactive intermediate species such as free radicals and hydroperoxides formed during lipid peroxidation are responsible for the production of toxic carbonyl compounds (Esterbauer, 1982). Therefore, it is important to measure the levels of toxic carbonyl compounds formed from biologically essential lipids such as blood plasma lipids and to search for a possible inhibitor of their formation. In the present study, the inhibitory effect of a flavonoid isolated from young green barley leaves (2″-*O*-glycosylisovitexin) toward glyoxal and malonaldehyde formation is investigated using cod liver oil and a blood plasma lipid, lecithin. The comparative study was performed using α-tocopherol.

MATERIALS AND METHODS

Chemicals. Quinoxaline, indole, 1,2-phenylenediamine hydrochloride, butylated hydroxytoluene (BHT), Trizma hydrochloride, and Trizma base were purchased from Sigma Chemical Co. (St. Louis, MO). α-Tocopherol, sodium dodecyl sulfate (SDS), hydrogen peroxide, 2-methylpyrazine, and Amberlite XAD-2 nonionic polymeric absorbent were bought from Aldrich Chemical Co. (Milwaukee, WI). 1,2-Dilinoleoyl-*sn*-glycero-3-phosphocholine (lecithin I) and 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine (lecithin II) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). *N*-Methylhydrazine (NMH) was obtained from Fluka Chemical Co. (Ronkonkoma, NY). Ferrous chloride was purchased from Fisher Scientific Co., Ltd. (Fair Lawn, NJ).

2″-*O*-Glycosylisovitexin (2″-O-GIV) was isolated from young green barley leaves (*Hordium vulgare L.* var. nudum Hook)

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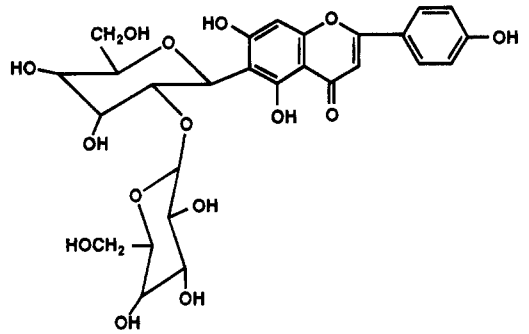

Figure 1. Structure of 2″-O-GIV.

harvested 2 weeks after germination by a method previously reported (Osawa et al., 1992) using column chromatography with Amberlite XAD-2 nonionic polymeric absorbent. The structure of 2″-O-GIV is shown in Figure 1.

Oxidation of Lecithins and Cod Liver Oil with Fenton's Reagent (Fe^{2+}/H_2O_2) in the Presence of 2″-O-GIV or α-Tocopherol. An aqueous solution (5 mL) containing 20 μL of cod liver oil or 5 mg (6.4 μmol) each of lecithins, 0.25 mmol of Trizma buffer (pH 7.4), 1 μmol of ferrous chloride, 0.5 μmol of hydrogen peroxide, 0.75 mmol potassium chloride, and 0.2% of surfactant SDS was stirred with various amounts of 2″-O-GIV or α-tocopherol for 16 h at 37 °C in a 20-mL test tube. The oxidation of samples was stopped by adding 50 μL of 4% BHT ethanol solution (Ichinose et al., 1989). The sample tubes were covered with aluminum foil during incubation to avoid any influence of light on the lipid peroxidation.

Analysis of MA. The gas chromatographic method for MA analysis reported previously (Ichinose et al., 1989; Dennis and Shibamoto, 1989) was used. The MA was reacted with NMH, and the resulting derivative, 1-methylpyrazole (1-MP), was analyzed with 2-methylpyrazine as an internal standard by a GC equipped with a fused silica capillary column and a nitrogen–phosphorus detector (NPD).

Analysis of Glyoxal. Glyoxal was analyzed as quinoxaline by a gas chromatograph (GC) with an NPD according to the method previously reported (Niyati-Shirkhodaee and Shibamoto, 1993) with a slight modification. After oxidation, 200 μL of aqueous 1,2-phenylenediamine hydrochloride (4.5 g/mL) solution was added to the samples. The sample solutions were stirred with a magnetic stirrer for 1 h at pH 7.5 and 25 °C. The reaction mixtures were mixed with 20 mL of saturated sodium chloride solution and then extracted with 15 mL of dichloromethane using a liquid–liquid continuous extractor for 3 h. After the extracts were dried over anhydrous sodium sulfate, their volume was adjusted to 10 mL with dichloromethane. Indole in 100 μL of dichloromethane solution (10 μmol/mL) was added to the samples as a gas chromatographic internal standard prior to analysis.

Instruments. A Hewlett-Packard (HP) Model 5880A GC equipped with an NPD and a 30 m × 0.25 mm i.d. bonded-phase DB-Wax fused-silica capillary column (J&W Scientific, Folsom, CA) was used for the quantitation of glyoxal and malonaldehyde. The GC peak areas were integrated with an HP 5880A integrator. The oven temperature was programmed from 120 to 220 °C at 10 °C/min and held for 20 min. Injector and detector temperatures were 250 °C and 300 °C, respectively. The linear velocity of the helium carrier gas was 42 cm/s with a split ratio of 21:1.

An HP 5890 GC interfaced to a VG Trio II mass spectrometer with a VG II-250 computer data system was used for mass spectrometric confirmation of the derivatives. The ionization voltage was 70 eV, and the ion source temperature was 150 °C. The column and oven conditions for GC/MS were as described for the GC analyses.

RESULTS AND DISCUSSION

MA is too unstable for direct measurement, which is a major limitation in analysis. To obtain quantitative values for MA, a stable derivative must be prepared.

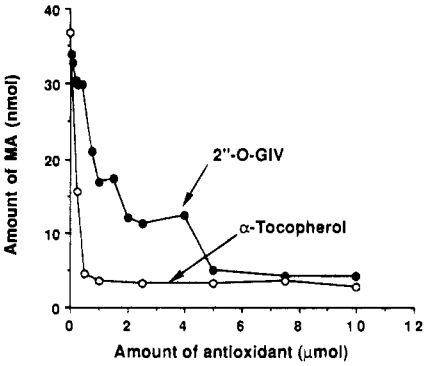

Figure 2. Effect of α-tocopherol or 2″-O-GIV on MA formation from lecithin I oxidized by Fenton's reagent.

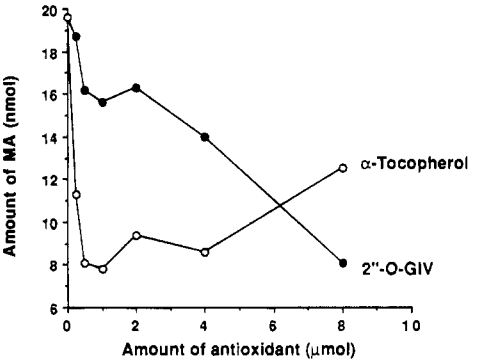

Figure 3. Effect of α-tocopherol or 2″-O-GIV on MA formation from lecithin II oxidized by Fenton's reagent.

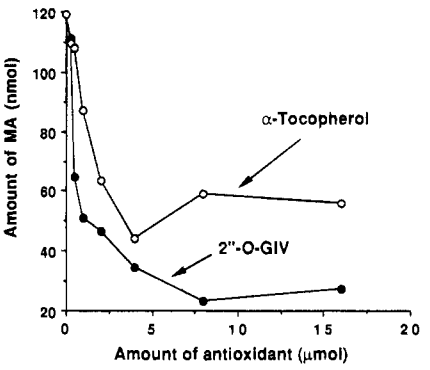

Figure 4. Effect of α-tocopherol or 2″-O-GIV on MA formation from cod liver oil oxidized by Fenton's reagent.

The most common method has been the TBA assay in which MA and TBA are reacted and the resulting red complex is measured colorimetrically. The method is, however, not specific for MA and its sensitivity is not sufficient for trace analysis of MA (Dennis and Shibamoto, 1989). Recently, we satisfactorily analyzed trace amounts of MA in lipids (Tamura and Shibamoto, 1991) and in liver microsomes (Ichinose et al., 1989) as the NMH derivative 1-MP using GC. The lowest detection level of 1-MP by NPD was 8.9 pg, equivalent to 7.8 pg of MA in the present study.

Figures 2, 3, and 4 show the inhibitory activity of 2″-O-GIV and α-tocopherol toward MA formation from oxidative degradation of lecithin I, lecithin II, and cod liver oil, respectively. The values are averaged from at least two replicate experiments. Both compounds exhibited dose-related activities. α-Tocopherol was very effective at low doses toward the two lecithins, inhibiting MA formation from lecithin I (Figure 2) and lecithin II (Figure 3) by 90% and 60%, respectively. In the case of lecithin II, however, its inhibitory effect decreased when

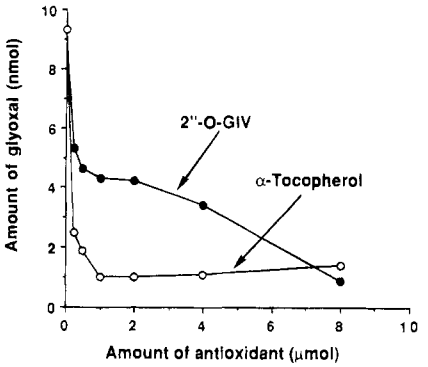

Figure 5. Effect of α-tocopherol or 2″-O-GIV on glyoxal formation from lecithin I oxidized by Fenton's reagent.

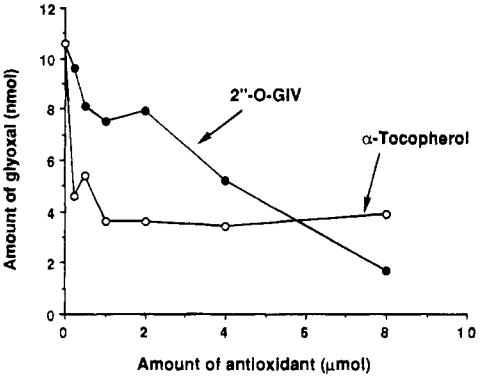

Figure 6. Effect of α-tocopherol or 2″-O-GIV on glyoxal formation from lecithin II oxidized by Fenton's reagent.

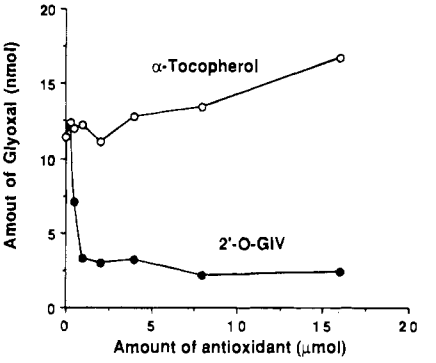

Figure 7. Effect of α-tocopherol or 2″-O-GIV on glyoxal formation from cod liver oil oxidized by Fenton's reagent.

the dose increased beyond 1 μmol. The same phenomenon was observed in the case of cod liver oil (Figure 4). 2″-O-GIV inhibited MA formation 85% at a level of 7.5 μmol in the case of lecithin I. Its effect was less than that of α-tocopherol at lower levels but became comparable at levels higher than 6 μmol. 2″-O-GIV exhibited a lesser effect than α-tocopherol when the level was lower than 6 μmol in the case of lecithin II. However, it became more effective when the dose increased above 6 μmol. In the case of cod liver oil, 2″-O-GIV showed a higher inhibitory effect than α-tocopherol throughout the levels tested. It inhibited MA formation by 81% from cod liver oil at a level of 8 μmol, whereas the highest effect obtained by α-tocopherol was 65% at a level of 4 μmol. Generally, α-tocopherol inhibited MA formation more effectively from the lipids with lower numbers of double bond than from those with higher numbers of double bond. On the other hand, 2″-O-GIV more effectively inhibited MA formation from a sample with higher numbers of double bond such as fish oil than from a sample with lower numbers of double bond such as lecithin I.

The formation of MA from ethyl ester of linoleic acid (fatty acid in lecithin I) was reportedly inhibited almost 100% in the presence of 4 and 3 μmol of 2″-O-GIV and α-tocopherol, respectively (Nishiyama et al., 1993). However, both compounds inhibited MA formation from ethyl ester of linolenic acid (fatty acid in lecithin II) by only 85% at optimum doses.

Analysis of trace glyoxal is also extremely difficult because it is not only highly reactive but also highly soluble in water. Therefore, derivatization is required for analysis. A quinoxaline produced from the reaction of glyoxal and *o*-phenylenediamine has been used successfully. For example, trace glyoxal was analyzed as a quinoxaline in cigarette smoke (Moree-Testa and Saint-Jalm, 1981). Recently, glyoxal formed from lipids and related compounds upon UV irradiation was determined using this derivative (Niyati-Shirkhodaee and Shibamoto, 1993). The lowest detection level of quinoxaline by NPD was 5.2 pg, equivalent to 2.3 pg of glyoxal in the present study.

Figures 5, 6, and 7 show the inhibitory activity of 2″-O-GIV and α-tocopherol toward glyoxal formation from oxidative degradation of lecithin I, lecithin II, and cod liver oil, respectively. The values are averaged from at least two replicate experiments. The trend of inhibition of glyoxal formation was quite similar to that of MA formation. In the case of lecithin I (Figure 5), α-tocopherol was very effective at low doses. The addition of 1 μmol of α-tocopherol to 5 mg of lecithin I inhibited the formation of glyoxal by 90%. The inhibitory activity of 2″-O-GIV was weaker than that of α-tocopherol at lower doses, but it was more effective at a higher dose. 2″-O-GIV inhibited the glyoxal formation by 90.3% at a dose of 8 μmol. In the case of lecithin II (Figure 6), 2″-O-GIV required 8 μmol to inhibit 84% of MA formation from 5 mg of lecithin II, whereas the maximum effect of α-tocopherol (68%) was obtained at a dose of 4 μmol. In the case of cod liver oil (Figure 7), α-tocopherol did not exhibit any inhibitory activity, but the formation of glyoxal increased slightly by the addition of α-tocopherol. On the other hand, 2″-O-GIV required only 1 μmol to inhibit 70% of glyoxal formation.

It is difficult to rationalize the decrease of inhibitory effect or even the prooxidative effect of α-tocopherol at higher doses in the case of cod liver system. The same phenomenon was observed when squalene was irradiated by UV light (Nishiyama et al., 1993). α-Tocopherol might be degraded into many products including MA and glyoxal at higher concentrations. However, further investigation is necessary to clarify this phenomenon.

The formation mechanisms of these aldehydes are not yet completely understood. The widely accepted formation mechanism of MA involves formation of a bicyclic endoperoxide intermediate (Pryor et al., 1976). This mechanism requires at least three methylene-interrupted double bonds for the formation of MA. Later, formation of MA from fatty acids with fewer than three double bonds was reported (Tamura and Shibamoto, 1991). Even though the presence of glyoxal in numerous materials such as ozonated drinking water (Glaze et al., 1989), cigarette smoke (Moree-Testa and Saint-Jalm, 1981), and heated glucose (Kasai and Nishimura, 1986) has been reported, the formation of glyoxal from lipids upon oxidative degradation was not reported until 1993 by Niyati-Shirkhodaee and Shibamoto, who found 70 nmol of MA formation from 1 mg of cod liver oil upon UV irradiation. Also, low molecular weight carbonyl compounds (including acetaldehyde, acrolein, propanal,

and acetone) reportedly produced glyoxal upon oxidative degradation by UV light. It was proposed that many low molecular weight radicals such as $^{\bullet}OH$, $^{\bullet}CHO$, $^{\bullet}CH_2CHO$, $^{\bullet}CH_3$, and $^{\bullet}COCH_3$ were formed from lipids by oxidation and then these radicals combined to form low molecular weight carbonyl compounds including MA and glyoxal.

As mentioned above, lipid peroxidation has been known to cause many pathological complications.

Although certain phospholipids reportedly inhibited Fe^{2+}/ascorbate-induced lipid peroxidation (Yoshida et al., 1993), the blood plasma phospholipids used in the present study clearly produced genotoxic MA and glyoxal. Use of antioxidants to prevent formation of reactive carbonyls in biological systems should be investigated further. In addition, if the reactive radicals mentioned above are formed during volatile aldehyde formation in blood plasma, investigation of their adverse effect is in order.

The antioxidative activity of 2″-O-GIV, which is comparative to that of α-tocopherol, was previously reported (Osawa et al., 1992). In the present study, 2″-O-GIV demonstrated a strong inhibitory effect toward glyoxal and MA formation from lipids. 2″-O-GIV may be useful as a food additive to prevent autoxidation of lipid-rich foods. Moreover, 2″-O-GIV is nontoxic and can be obtained in large quantities from a natural source at low cost (Osawa et al., 1992).

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