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Interaction between flavonoids and α-tocopherol in human low density lipoprotein

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Oxidative modification of low density lipoprotein (LDL) may play an important role in the development of atherosclerosis. α-Tocopherol functions as a major antioxidant in human LDL. The present study was to test whether four natural flavonoids (kempferol, morin, myricetin, and quercetin) would protect or regenerate α-tocopherol in human LDL. The oxidation of LDL incubated in sodium phosphate buffer (pH 7.4, 10 mM) was initiated by addition of either 5.0 mM $CuSO_4$ at 37°C or 1.0 mM of 2,2′-azo-bis (2-amidinopropane) dihydrochloride (AAPH) at 40°C. It was found that α-tocopherol was completely depleted within 1 hour. Under the same experimental conditions, all four flavonoids demonstrated a dose-dependent protecting activity to α-tocopherol in LDL at the concentration ranging from 1 to 20μM. All flavonoids showed a varying protective activity against depletion of α-tocopherol in LDL, with kempherol and morin being less effective than myricetin and quercetin. The addition of flavonoids to the incubation mixture after 5 minutes demonstrated a significant regeneration of α-tocopherol in human LDL. The protective activity of four flavonoids to LDL is related to the number and location of hydroxyl groups in the B ring as well as the stability in sodium phosphate buffer. (J. Nutr. Biochem. 11:14–21, 2000)

Keywords: kempferol; morin; myricetin; quercetin; α-tocopherol; low density lipoproteins; stability

Introduction

The oxidation of low density lipoproteins (LDL) may play an important role in the development of atherosclerosis.[1–7] α-Tocopherol (the major form of vitamin E) is a primary antioxidant that protects LDL from oxidation.[8,9] Increased vitamin E intake has been associated with the reduced oxidation of LDL, platelet adhesives, and thrombosis.[10–15] Epidemiologic data also suggest that the vitamin E supplement is associated with a lower risk of coronary heart disease in both men and women.[16–18] Growing evidence for the protective effect of vitamin E in humans also includes an inverse association between plasma α-tocopherol and mortality from cardiovascular heart disease.[19–20] Ascorbic acid is also widely accepted as an antioxidant in vivo both directly or indirectly in maintaining and regenerating α-tocopherol.[21–23] In fact, the level of plasma ascorbic acid is inversely correlated with coronary heart disease mortality.[24]

Fruits, vegetables, and some plants used in herbal medicines are rich sources of antioxidants, which are mainly flavonoids. The daily intake of these flavonoids in humans is estimated to be as much as 1 g.[25,26] It has been shown that flavonoids possess antioxidant activity to LDL in vitro.[27–29] Hertog et al.[30] found an inverse correlation between flavonoid consumption and coronary heart disease mortality. The chemistry, metabolism, and health benefits of flavonoids have been recently reviewed by Cook and Samman.[31]

More evidence is accumulating that flavonoids can be

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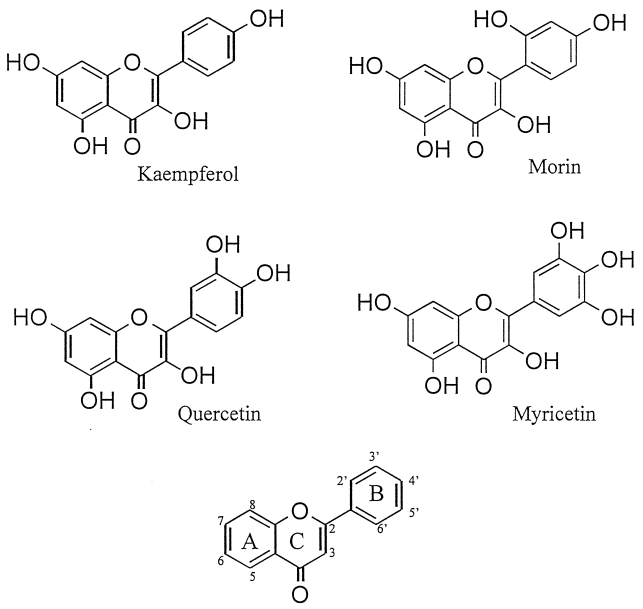

Figure 1 Chemical structures of kempferol, morin, myricetin, and quercetin

absorbed and circulated in human plasma.[32–34] However, the data addressing the interaction between the flavonoids and α-tocopherol in LDL are very limited. The four most common flavonoids (quercetin, myricetin, kempferol, and morin) are characterized by sharing a similar backbone with varying number and location of hydroxyl groups (*Figure 1*). The present study was designed to examine (1) the function of location and number of hydroxyl groups in their anti-LDL oxidation activity, (2) the protective role of four flavonoids to α-tocopherol in LDL, (3) the regenerating activity of α-tocopherol of four flavonoids in LDL, and (4) the stability of these four flavonoids in relation to their antioxidant activity.

Material and methods

Flavonoids

Kempferol, morin, quercetin, myricetin, and apigenin were obtained from Sigma Chemical Co. (St. Louis, MO USA). Their purity was checked using both thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). TLC analysis was performed using two developing solvent systems (chloroform:methanol:acetic acid, 20:2:1, vo/vol/vol; butanol:water:acetic acid, 4:1:1, vol/vol/vol), and only one single dot was visualized under ultraviolet light. The HPLC analysis as described below showed a single peak, indicating the high purity of these flavonoids.

HPLC analysis of flavonoids

Purity and stability of the flavonoids were determined using a Shimadzu LC-10AD HPLC (Tokyo, Japan) equipped with a ternary pump delivery system. In brief, 15 μL of the pure flavonoids or mixtures (2 mg each/mL) was injected onto a column (Hypersil ODS, 250 × 4.6 mm, 5 μm; Alltech, Deerfield, IL USA) using a rheodyne valve (20 μL capacity; Rheodyne, Cotati, CA USA). A gradient of phosphoric acid solution (0.01 M) and methanol was used at a flow rate of 0.6 mL/min (0–55 min, 30% methanol changing to 45%; 55–95 min, 45% methanol changing to 100%; 95–100 min, 100% methanol changing to 30%). The column temperature was set at 40°C in a water bath. The individual flavonoids were separated and quantified using an ultraviolet detector at 285 nm (UVIS-205, Alltech).

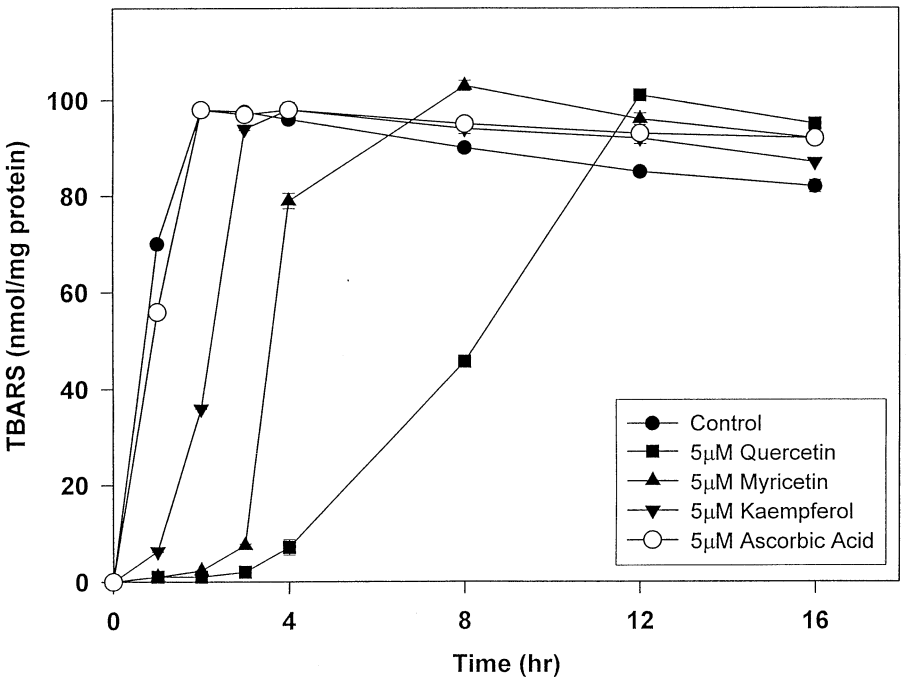

Figure 2 Inhibitory effect of four flavonoids on production of thiobarbituric acid reactive substances (TBARS) in Cu^{++}-mediated oxidation of human low density lipoprotein (LDL). The LDL (150 μg protein/mL) was incubated in sodium phophate buffer (pH 7.4) containing 5 mM $CuSO_4$ at 37°C. Data are expressed as means ± SD of five samples.

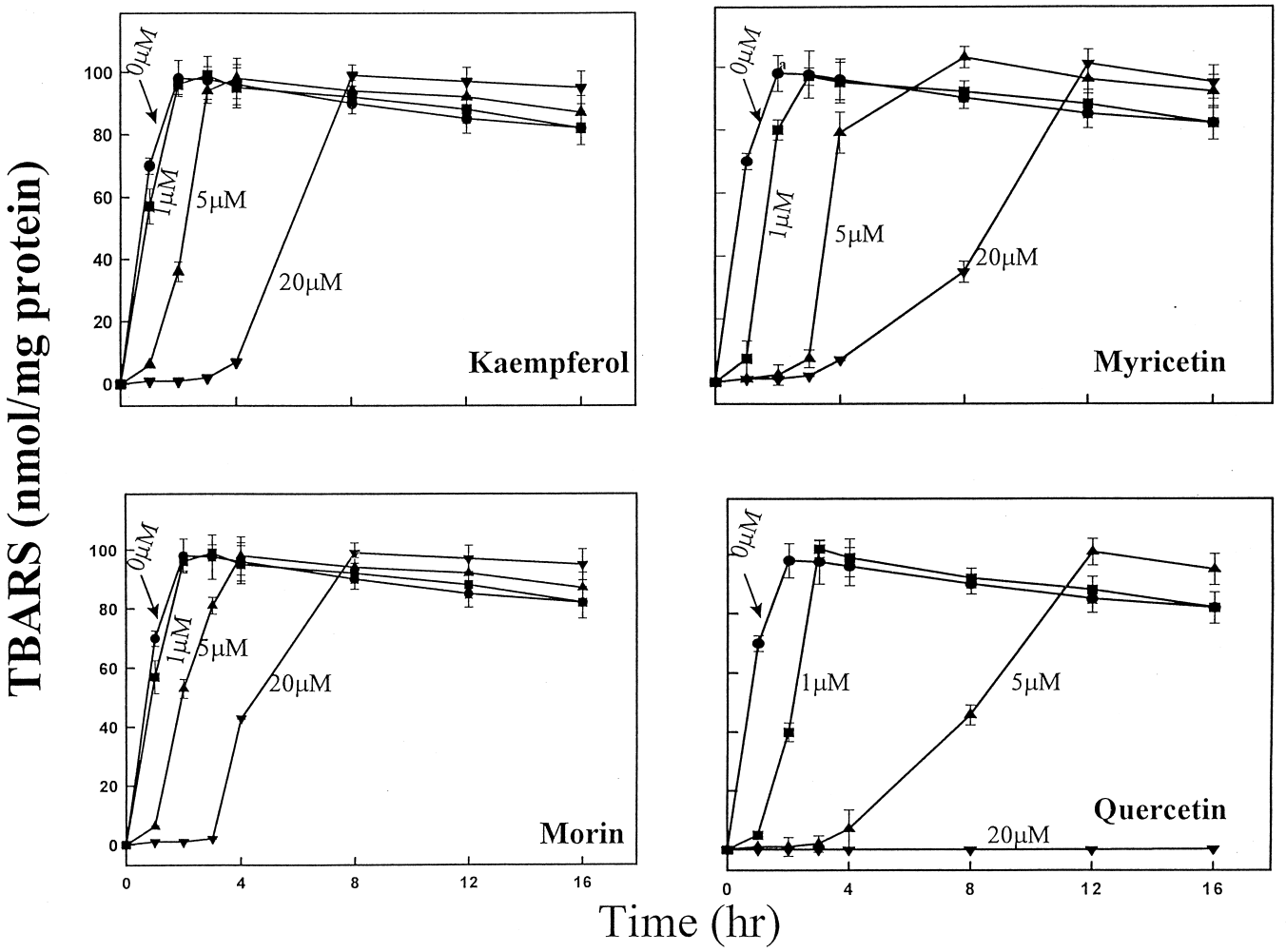

Figure 3 Dose-dependent inhibitory effect of kempferol, morin, myricetin, and quercetin on production of thiobarbituric acid reactive substances (TBARS) in Cu^{++}-mediated oxidation of human low density lipoproteins (LDL). The LDL (150 μg protein/mL) was incubated in sodium phophate buffer (pH 7.4) containing 5 mM $CuSO_4$ at 37°C. Data are expressed as means ± SD of five samples.

LDL isolation

Fresh plasma was collected and pooled from healthy subjects ($n = 20$) at the Prince of Wales Hospital (The Chinese University of Hong Kong, Shatin, Hong Kong, China). EDTA (0.1%) and NaN_3 (0.05%) solutions were immediately added to prevent the lipoprotein from oxidative modification. LDL was isolated from plasma according to the method described previously.[35] Briefly, the plasma was first centrifuged at 1,500 × g for 15 minutes to remove cells and cell debris. The density was then increased to 1.019 by addition of a NaCl-KBr solution (153 g NaCl, 354 g KBr, and 100 μg EDTA dissolved in 1 L of H_2O; density = 1.33 g/mL) and recentrifuged at 160,000 × g at 4°C for 20 hours. After removing the top layer containing chylomicron and very low density lipoprotein, the density of remaining plasma fractions was increased to 1.064 and recentrifuged at 160,000 × g for an additional 24 hours. The top LDL fraction was collected and then flushed with nitrogen and stored at −70°C. The protein content of isolated LDL was determined using Lowry's method.[36] The total LDL cholesterol and triacylglycerols were measured using Sigma enzymatic kits. The LDL fraction contained 5.0 mg protein, 2.2 mg cholesterol, and 0.9 mg triacylglycerols per milliliter.

LDL oxidation

The stock LDL fraction (5 mg protein/mL) was dialyzed against 100 volumes of the degassed dialysis solution (pH 7.4) containing 0.01 M sodium phosphate, 0.9% NaCI, 10 μM EDTA, and 0.05% NaN_3 in dark for 24 hours. The dialysis solution was changed four times. Oxidation of LDL was conducted as previously described by Pahl et al.[37] In brief, 100 μg LDL protein was incubated in a mixture containing 5 mM $CuSO_4$ and varying concentrations of individual flavonoids at 37°C. The oxidation was then stopped by addition of 25 mL 1.0% EDTA and cooled at 4°C. The degree of LDL oxidation was monitored by measuring the production of thiobarbituric acid reactive substances (TBARS) as previously described by Buege and Aust[38] The LDL-incubated tube was added immediately to

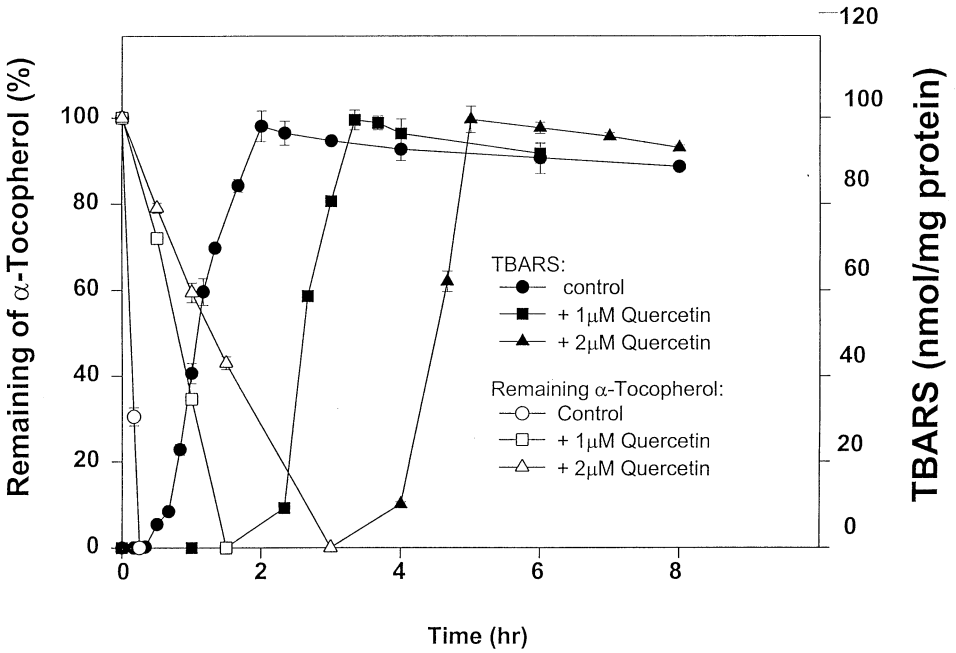

Figure 4 Relationship between the remaining α-tocopherol (initial concentration = 1.5 μM) in human low density lipoproteins (LDL) and the production of thiobarbituric acid reactive substances (TBARS). The LDL (150 μg protein/mL) was incubated in sodium phosphate buffer (pH 7.4) containing 5 mM $CuSO_4$ with or without addition of 1 μM and 2 μM quercetin. The oxidation of LDL was conducted at 37°C. The production of TBARS and the remaining α-tocopherol in LDL were measured periodically at the same time. Data are expressed as means ± SD of five samples.

2 mL of 0.67% thiobarbituric acid and 15% trichloroacetic acid in 0.1 N HCL solution. The incubation mixture was then heated at 95°C for 1 hour, cooled on ice, and centrifuged at 1,000 × g for 20 minutes. TBARS were then determined by measuring the absorbance at 532 nm. The calibration was done with a malondialdehyde (MDA) standard solution prepared from tetramethoxylpropane. The value of TBARS was expressed as nmol MDA per milligram LDL protein.

Depletion of α-tocopherol in LDL

The stock LDL was dialyzed as described above. Oxidation of α-tocopherol in LDL was induced by either 5 mM $CuSO_4$ at 37°C or 1 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) at 40°C. In brief, the LDL (150 μg protein/mL) was incubated in sodium phosphate buffer (pH 7.4, 10 mM) with constant stirring. The varying amounts of flavonoids were added either before or 5 minutes after addition of 1.0 mM AAPH. An aliquot of the incubation solution (1 mL) was taken periodically and chilled in ice. After immediate addition of 1 mL of ethanol containing 0.5 mg of butylated hydroxyltolune as an antioxidant and 1.0 μg of tocopherol acetate as an internal standard, the mixture was immediately extracted using 2 mL of hexane. The hexane was evaporated under a gentle stream of nitrogen and the resulting extracts were redissolved into 100 μL of ethanol followed by HPLC analysis for determination of the remaining α-tocopherol in LDL.

HPLC analysis of α-tocopherol in LDL

α-Tocopherol in LDL was determined as we described previously[39] using a Shimazu LC-10AD HPLC (Tokyo, Japan) equipped with a ternary pump delivery system. In brief, 10 μL of the extracts in ethanol derived from 1 mL of the LDL incubation solution was injected onto a C-18 column (Microsorb MV, 250 × 4.6 mm inner diameter, 5 μm; Rainin, Woburn, MA USA) using a rheodyne valve (20 μL capacity; Rheodyne, Cotati). Methanol was used as an eluting solvent at a flow rate of 2 mL/min. α-Tocopherol was monitored using a diode detector at either 200 nm or 280 nm and quantified according to the amount of α-tocopherol acetate added. The initial concentration of α-tocopherol in the final LDL incubation solution was found to be 1.5 μM (0.65 μg/mL).

Stability of flavonoids

The antioxidant activity of a flavonoid may be partially dependent on its stability in the medium chosen. To explain the varying effectiveness of four flavonoids against α-tocopherol depletion in LDL, the stability of these flavonoids (0.5 mM each) was examined in sodium phosphate buffer (10 mM, pH 7.4). An aliquot (0.4 mL) of the incubation mixture was periodically sampled, and 0.1 mL of apigenin solution (0.5 mg/mL) was added as an internal standard. The mixture was subjected to HPLC analysis as described previously.

Results

Kempferol, morin, myricetin, and quercetin showed varying effectiveness in inhibiting the production of TBARS of LDL incubated in the presence of 5 mM $CuSO_4$ (*Figure 2*). Quercetin was most effective followed by myricetin, whereas kempferol and morin were very similar in protecting the LDL from oxidation. Ascorbic acid showed no or little protection to LDL from Cu^{++}-catalyzed oxidation (*Figure 2*). The antioxidative effect of the four flavonoids tested on oxidation of LDL appeared to be dose dependent (*Figure 3*).

The production of TBARS in LDL was examined in

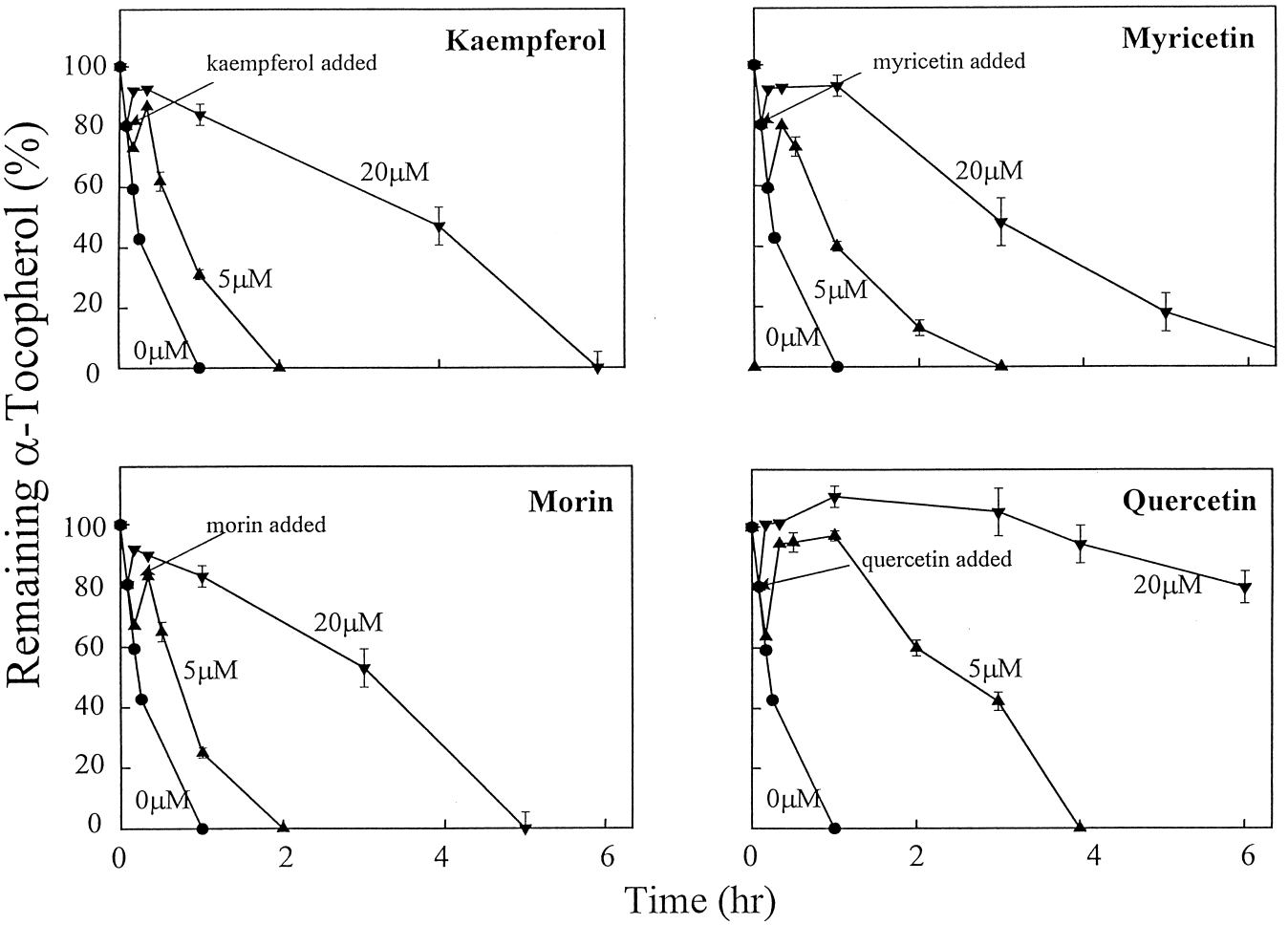

Figure 5 Time course of α-tocopherol regeneration in human low density lipoproteins (LDL) by flavonoids. The LDL (150 μg protein/mL) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol (initial concentration = 1.5 μM) in LDL was induced by 1 mM of 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH) at 40°C. Values are means ± SD of five samples.

relation to the concentration of LDL α-tocopherol. Depletion of α-tocopherol in LDL was initiated by addition of 1 mM AAPH.[38] The data on the four flavonoids demonstrated that LDL oxidation (TBARS production) did not occur substantially until α-tocopherol was depleted (*Figure 4*). To simplify the presentation, only data on quercetin were shown.

Kempferol, morin, myricetin, and quercetin added to the incubation medium delayed the depletion of α-tocopherol (*Figure 4*). The inhibitory effect of the four flavonoids on α-tocopherol consumption was dose dependent. Similarly, quercetin was most effective followed by myricetin, morin, and kempferol (*Figure 4*).

To examine whether they can regenerate α-tocopherol from its free radical or quinoid form, four flavonoids at varying concentrations were added 5 minutes after the α-tocopherol depletion reaction had been initiated. The initial concentration of α-tocopherol was 1.5 μM in the incubation medium. As shown in *Figure 5*, α-tocopherol in LDL decreased by addition of 1.0 mM AAPH and then recovered to some extent following the addition of varying concentrations of kempferol, morin, myricetin, and quercetin (*Figure 5*).

To partially explain the relative effectiveness in protecting LDL against oxidation, the stability of four flavonoids was examined in phosphate buffer (*Figures 6 and 7*). Myricetin was most unstable and was completely depleted within the first hour. For the same period, only 20% of quercetin was depleted. Under the same conditions, kempferol was relatively stable followed by morin.

Discussion

Flavonoids are regular components in the human diet. They are mainly derived from consumption of vegetable, fruits, tea, and wine. Together with previous studies,[28,29] the present results clearly showed that flavonoids strongly inhibit LDL oxidation (*Figures 2 and 4*). They probably protect LDL from oxidative modification by one or more of the following possibilities: (1) Flavonoids may function as primary antioxidant by directly reducing the formation of free radicals mediated by Cu^{+2} and AAPH[40]; (2) they may spare, maintain, and regenerate α-tocopherol (*Figure 5*); or (3) they may function as chelators to inactive Cu^{+2} and other cations involved in intiation of free radicals (*Figures 2 through 4*).

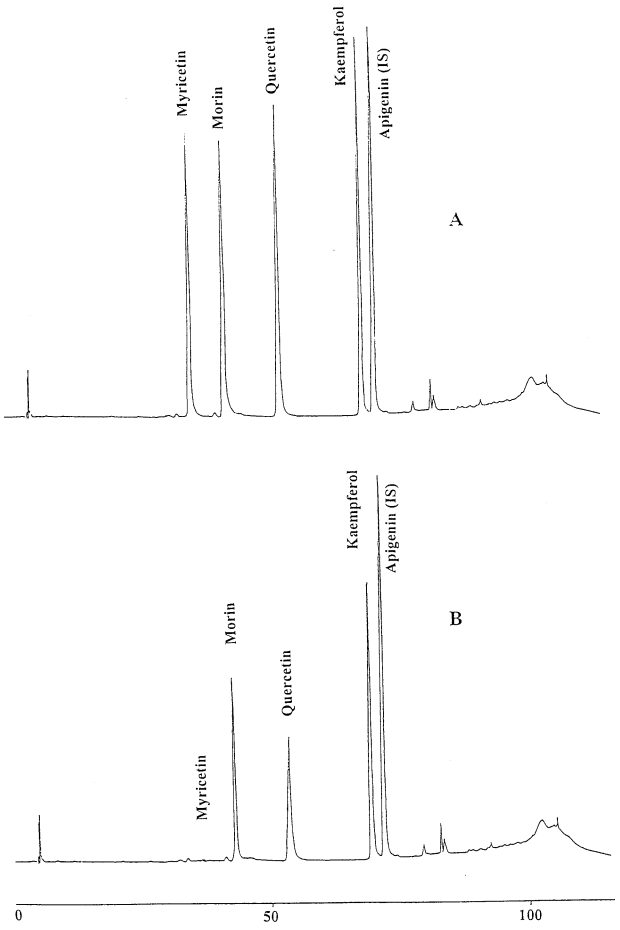

Figure 6 High performance liquid chromatograms showing the stability of 0.5 mM kempferol, morin, myricetin, and querecetin in sodium phosphate buffer (pH 7.4). (*A*) 0 hour; (*B*) 1 hour.

The regenerating α-tocopherol property is not unique to flavonoids. Ascorbic acid, glutathione (GSH), and green tea epicatechins have been also shown to be capable of regenerating α-tocopherol in a number of systems.[21,22,39,41,42] To our best knowledge, the present study was the first to demonstrate that flavonoids also possess α-tocopherol-repairing activity in human LDL under the present experimental conditions. It is known that a mixture containing both water-soluble and fat-soluble antioxidants will be more effective than a single antioxidant because the former mixture is capable of quenching free radicals in both aqueous and lipid phases.[43] Therefore, anti-free radical function of α-tocopherol can be augmented in LDL when any water-soluble antioxidant, particularly ascorbic acid and possibly flavonoids, is present due to their regenerating activity of α-tocopherol.

There is convincing evidence that flavonoids can be absorped and circulated.[32–34] Hollman et al.[44] showed that the maximum plasma concentration of quercetin and its metabolites could reach 200 ng/mL. This study also showed that quercetin may accumulate in plasma with repeated dietary intake. The present results, although not directly transferable to in vivo conditions, may have implications for individuals who consume diets rich in flavonoids daily. In this connection, the ingestion of red wine flavonoids has been shown to be associated with a significant decrease in human LDL cholesterol and LDL oxidation.[28,45] In another crossover study in which 180 mL wine was given to the individual for 4 weeks, the results showed that white wine can decrease the TBARS of the plasma lipid significantly.[46] If consumption of flavonoids is associated with a lower risk of coronary heart disease,[30] part of the mechanism may involve an increase in plasma antioxidant capacity either by directly protecting LDL from oxidation[28,29] or by maintaining and regenerating α-tocopherol in LDL.

The four flavonoids demonstrated varying protective effects on human LDL (*Figure 2*). The relative ineffectiveness of kempferol in comparison to myricetin and quercetin was related to the number and position of their aromatic hydroxyl groups. As shown in *Figure 4*, kempferol only has one hydroxyl group on the B ring whereas quercetin and myricetin have two or more hydroxyl groups on the B ring, indicating that a minimum of two hydroxyl groups on the B ring are necessary for the antioxidant activity. When the antioxidant activity of quercetin was compared with that of morin, the antioxidant role of two adjacent hydroxyl groups was best illustrated. This is consistent with the study by Bors and Saran[46] who reported that o-dihydroxyl structure in the B ring conferred stronger antioxidant activity. Perhaps the two adjacent hydroxyl groups at positions 3′ and 4′ in quercetin were more vulnerable to loss of a proton than the two hydroxyl groups at positions 2′ and 4′ in morin due to stability of the radical intermediate and the resonance delocalization. For the same reason, the antioxidant activity of myricetin with three adjacent hydroxyl groups should be stronger than quercetin. However, the reverse was observed (*Figure 2*). The relative ineffectiveness of myricetin against LDL oxidation compared with that of quercetin can be

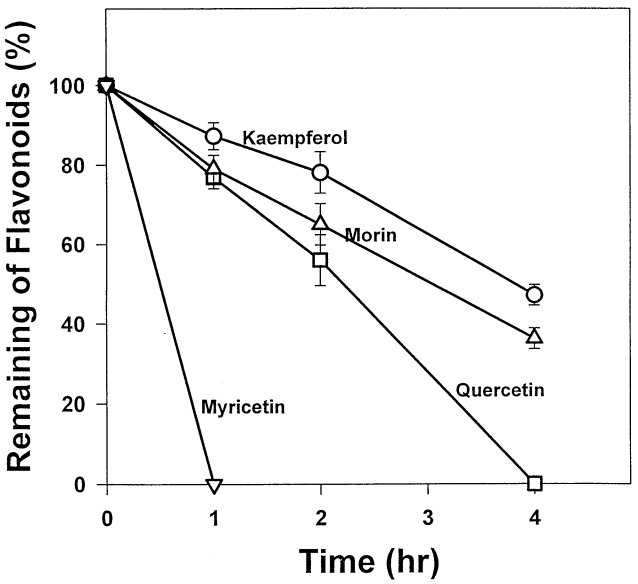

Figure 7 Stability of individual flavonoids (0.5 mM) in sodium phosphate buffer (pH 7.4). The flavonoids were measured using high performance liquid chromatography as described in the text. Values are means ± SD of five samples.

explained by the observation that the former was more unstable than the latter in the phosphate buffer (*Figures 6 and 7*). It is in agreement with the report by Zhu et al.,[47] who showed that the three adjacent hydroxyl groups in (-)-epigallocatechin and (-)-epigallocatechin gallate were much more unstable than the two adjacent hydroxyl groups in (-)-epicatechin and (-)-epicatechin gallate. The lack of effectiveness of myricetin also may be attributed to its known pro-oxidant and antioxidant behavior.[48] It is well known that many phenolic compounds are potent antioxidants but they may also act as pro-oxidants in some systems.[49]

In conclusion, the present results demonstrated that flavonoids function as antioxidants, protecting LDL from oxidation either directly or indirectly via the mechanism of maintaining and regenerating α-tocopherol in LDL. The effectiveness of a flavonoid in protecting LDL from oxidation is governed by its stability, number, and location of hydroxyl groups.

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