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ARTICLE

Low-Density-Lipoprotein (LDL)-Bound Flavonoids Increase the Resistance of LDL to Oxidation and Glycation under Pathophysiological Concentrations of Glucose in Vitro

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The higher susceptibility of low-density lipoprotein (LDL) to oxidation and glycation in diabetes has been shown to be related to poor glycemic control. The aim of this study was to determine whether LDL-bound flavonoids attenuate high-glucose (HG)-mediated LDL oxidation and glycation. For this purpose, human plasma was preincubated with individual flavonoids for 3 h, followed by sequential ultracentrifugation and extensive dialysis to remove unbound flavonoid samples. Enriched LDL was subsequently isolated and challenged for its resistance to oxidation and glycation. Results showed that glucose (5−30 mM) dose-dependently accelerates copper (Cu^{2+})-mediated LDL oxidative modification. The enrichment of flavonoids such as luteolin, naringenin, and kaempferol significantly increased the resistance of LDL to oxidation and prevented endogenous α-tocopherol consumption caused by HG/Cu^{2+} ($p < 0.05$). The long-term glycation of LDL, which was measured by advanced glycation endproducts (AGEs)-related fluorescence and boronate affinity chromatography, was found to be inhibited by LDL-bound flavonoids in the following order: rutin > luteolin > quercetin > kaempferol > naringenin > catechin ≈ EC > naringin. Moreover, a solid-phase extraction system with HPLC−diode array detection provided evidence that flavonoids were bound to LDL particles to a certain extent concurrently facilitating the lipoprotein antioxidant and antiglycation activities. In conclusion, this study supports the hypothesis that HG promoted oxidative and glycative modifications of LDL. This is the first study to show that the introduction of flavonoids into LDL particles protects the lipoprotein against glycotoxin-mediated adverse effects.

KEYWORDS: Advanced glycation endproducts; binging; flavonoids; glucose; glycation; LDL; oxidation; tocopherol

INTRODUCTION

According to the World Health Organization, more than 171 million of people are afflicted by diabetes mellitus worldwide, and the incidence may double between 2000 and 2030. Diabetes is a chronic metabolic disease associated with poor glycemic control. Physiologically, long-term high glucose can lead to heart, kidney, eye, nerve, and vascular tissue disorders. Many studies have demonstrated that people with diabetes have a 2−4-fold increased risk of developing cardiovascular disease, which is responsible for ca. 50% of deaths among people with diabetes (*1*). The mechanisms behind this elevated risk are still not fully understood, although there is now increasing evidence for a role of oxidative and glycative/glycoxidative modifications of low-density lipoprotein (LDL) induced by hyperglycemia (*2*, *3*). It has been suggested that these modifications may have a common element in the overproduction of reactive oxygen species (ROS) during reactions.

In diabetes-associated atherosclerosis, both glycation and oxidative processes have been implicated in disease development and progression. Previous work suggested that LDL oxidizability may be increased in people with diabetes and that glycated lipoproteins in such individuals are particularly high when glycemic control is poor (*3*). Glycation of proteins involves reactions of sugar, such as glucose or another reactive aldehyde, on the protein. The extent of this spontaneous reaction is dependent on the duration of exposure to the modifying species and results in the generation of a heterogeneous group of adducts known as advanced glycation endproducts (AGEs). Notably, glucose and other aldehydes, whether free or protein-bound, can also undergo autoxidation reactions, which yield radicals and other reactive intermediates (e.g., H_2O_2 and other peroxides), which can also contribute to AGEs formation. These latter processes are often termed glycoxidation (*4*).

It has been known that glucose-mediated modification of LDL is a rather complex process during which chemical, structural, and biofunctional properties of the LDL lipids and proteins progressively change (*2*). These reactions can be nonoxidative (glycation) or oxidative (glycoxidation) and result in the conversion of LDL to a form that is recognized by the scavenger receptors of macrophages (*3*). Another atherogenic effect of glycation is

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related to an increased susceptibility to LDL oxidation. The higher incidence of atherosclerosis in type 2 diabetics has been related to the increased susceptibility of glycated LDL to oxidation (*5*). Even under hypoxic conditions, or in the absence of free radicals, ROS could still be generated from glucose or Amadori products via glycation reaction, further promoting glycated LDL oxidation (*6*). Additionally, two studies have shown that high glucose (HG) increases the susceptibility of LDL to oxidation (*7*, *8*). Therefore, oxidative damage caused by HG is considered to be a necessary step along the path to atherosclerosis.

Flavonoids are present in various kinds of vegetables, tea, and red wine. The primary structure of flavonoids is three benzene rings with one or more hydroxyl groups. This is also the key factor that determines their antioxidation strength. The antioxidant activity of flavonoids has been demonstrated in many lipid systems; therefore, they are speculated to have potential in atherosclerosis prevention (*9*). In vitro, flavonoids inhibit oxidation of LDL by macrophage-mediated or cell-free systems (*10*). Antioxidation may involve the ability of flavonoids to scavenge free radicals, chelation of transition metal ions, sparing of LDL-associated antioxidants, and binding to macromolecules or interaction with other kinds of antioxidants (*11*−*13*).

In the present study, we first confirmed that the pathological concentrations of glucose promote oxidative modification of LDL in vitro. We then investigated whether LDL-bound flavonoids could inhibit LDL oxidation in a HG/Cu^{2+} pro-oxidation system and glycation in a long-term HG-incubated model. The aim of this study was to determine whether naturally occurring flavonoids could play an advantageous role in preventing or relieving diabetic complications such as atherosclerosis.

MATERIALS AND METHODS

Chemicals. Catechin, epicatechin (EC), kaempferol, quercetin, rutin, luteolin, naringin, naringenin, D-glucose, and α- and γ-tocopherols were purchased from Sigma Chemical Co. (St Louis, MO). Protein assay kit and chemicals for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Laboratories (Hercules, CA). Paragon Lipo agarose-gel electrophoresis kit was purchased from Beckman Co. (Fullerton, CA). *m*-Aminophenyl boronate gel was purchased from Pierce Chemical Co. (Rockford, IL). Waters Oasis HLB extraction cartridge was purchased from Waters (Milford, MA). All other chemicals were of analytical grade and all solvents were of high-performance liquid chromatography (HPLC) grade from Merck (Darmstadt, Germany).

Test of Antioxidant Protection in Vitro. Blood from six healthy male volunteers (age range from 20 to 28 years) on diets without flavonoids supplementation for 3 days was collected in tubes containing EDTA (1 mg/mL) after an overnight fast. Plasma was isolated by centrifugation at 4 °C. To investigate whether flavonoids provide antioxidant protection in vitro, pooled plasma was preincubated with an ethanolic solution of flavonoids to a final concentration of 100 μmol/L for 3 h at 37 °C under nitrogen in the dark, as described by Esterbauer et al. (*14*). α-Tocopherol (100 μmol/L) and the same amount of pure ethanol (0.1% of total plasma volume) were used as positive and negative controls, respectively.

LDL Isolation. Native or antioxidant-enriched LDL (d = 1.019−1.063 g/mL) was isolated from plasma by sequential density gradient ultracentrifugation (Himac CS 120GX, Hitachi, Tokyo, Japan). The LDL fraction was extensively dialyzed three times for 4, 4, and 16 h against 1000-fold excess of phosphate-buffered saline (PBS; 10 mmol phosphate buffer/L, 150 mmol NaCl/L, pH 7.4) containing 10 μmol/L EDTA at 4 °C in the dark and filtered through a 0.22 μm filter. Just before experiments, LDL was dialyzed twice (for 4 h and then 16 h) against 5 L of PBS to remove EDTA and unbound flavonoids. Protein concentration was determined using a Bio-Rad assay kit with bovine serum albumin as a standard. Unless specifically stated in the text, these LDL preparations were used within 1 week.

Oxidation of LDL with Cu^{2+} in the Presence of Different Concentrations of Glucose. LDL was diluted with PBS up to a concentration of 100 μg of protein/mL and incubated at 37 °C with freshly prepared $CuSO_4 \cdot 5H_2O$ (2.4 μmol/L) and glucose (0, 5, 15, and 30 mM). Aliquots were tested to determine the effect of flavonoids on the formation of conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARs) due to LDL oxidation, fragmentation of ApoB, relative electrophoretic mobility (REM), and the consumption of endogenous antioxidants in LDL.

CD Formation. Continuous monitoring of LDL conjugated diene formation was performed according to the method described by Esterbauer et al. (*15*). Briefly, 2 mL of LDL (100 μg of protein/mL) was incubated at 37 °C in a motorized six-cuvette temperature-controlled Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan). Absorbance at 234 nm was recorded every 5 min. The lag phase was determined from the intersection of two lines showing changes in optical density: the first line corresponds to the utilization of endogenous antioxidants in the LDL, and the second line corresponds to the subsequent rapid rise in LDL oxidation following the exhaustion of endogenous or exogenous antioxidants.

Fluorometric Measurement of TBARs. The formation of TBARs in the LDL sample was measured according to the method described by Yagi (*16*). LDL was mixed with 50 μL of BHT (4% w/v ethanol), 500 μL of sodium dodecyl sulfate (0.3%), 2 mL of 0.1 N HCl, 0.5 mL of phosphotungstic acid (10%), and 1 mL of thiobarbituric acid (0.7%). After heating at 100 °C for 45 min, the fluorescent reaction products were assayed using a spectrofluorometer (Hitachi, 650-40) with excitation at 515 nm and emission at 553 nm. The concentration of TBARs was expressed as equivalents of 1,1,3,3-tetraethoxypropane, which was used as the standard.

REM. The degree of LDL oxidation was determined using the Poragon Lipo agarose-gel electrophoresis kit (Beckman, Brea, CA) according to the manufacturer's instructions. Briefly, 4 μL of each sample was applied to the Lipo gel (0.5% agarose gel) slot. The electrophoresis was performed at 100 V for 30 min in a barbital buffer, after which the gel was fixed for 10 min and completely dried. The dried gel was subsequently stained with Sudan Black and then destained. The increased electrophoretic mobility of LDL was expressed relative to the mobility of native LDL.

SDS-PAGE. Apolipoprotein B (ApoB) fragmentation was performed by SDS-PAGE. LDL was dissolved in a sample buffer (containing 0.15% SDS, 0.25% glycerol, 0.2 M Tris-HCl, pH 6.8, and 6% β-mercaptoethanol), mixed, and incubated in boiling water for 5 min. For each sample, 15 μL was loaded onto a 5% SDS-PAGE gel, and electrophoresis was performed at a constant current of 200 V for 45 min at 4 °C. Gels were stained with Coomassie Blue R-250.

Assay of α- and γ-Tocopherols in LDL. α- and γ-tocopherols were extracted from LDL and analyzed using the method described by Lang et al. (*17*). An aliquot of LDL (100 μg of protein/mL) was precipitated with ethanol, which contained α- and γ-tocopherol acetate as internal standards, and was subsequently extracted with hexane. The hexane upper phase was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in an alcohol solution (ethanol/isopropanol 95:5, v/v) and separated by HPLC. HPLC analysis was performed using a Hitachi liquid chromatograph, consisting of a model L-6200 pump, a Rheodyne model 7125 syringe-loading sample injector, a model D-2000 integrator, and a diode array detector (Hitachi, L-7455 model) set at 292 nm. Each sample (10 μL) was injected onto a LiChrospher 100 RP-18 (5.0 μm) column (Merck) and eluted by a mixture of methanol/alcoholic solution (65:35, v/v) at room temperature, with a flow rate of 1.5 mL/min.

Determination of LDL Glycation. Glycation of LDL was performed by incubating native or antioxidant-enriched LDL with 30 mM glucose in PBS (pH 7.4) containing 0.02% sodium azide at 37 °C for 6 weeks under sterile conditions. The degree of LDL glycation was detected by affinity gel chromatography with *m*-aminophenyl boronate gel, as described by Chang et al. (*18*). Briefly, Bio-Rad Poly-Prep Chromatography Columns (Bio-Rad, Hercules, CA) packed with 1 mL of the gel were prepared and equilibrated with 5 mL of binding buffer (200 mmol/L ammonium acetate, pH 8.8). After equilibration, LDL aliquots (300 μg of protein/mL) were added and allowed to soak into the gel, followed by 5.5 mL of the equilibration buffer. The outflow tubing of the column was changed, and the glycated LDL fraction was eluted from the gel column by adding 2 mL of elution buffer (HCl, 25 mmol/L). The relative degree of LDL glycation was spectrophotometrically measured by determining the

absorbance at 414 nm, and the degree of glycation (glycation %) was calculated according to the following equation:

$$\text{glycation\%} = \left[\frac{\text{OD}_{414}\text{ of glycated LDL} \times 2}{\text{OD}_{414}\text{ of glycated LDL} \times 2 + \text{OD}_{414}\text{ of nonglycated LDL} \times 6}\right] \times 100\%$$

Detection of Flavonoid Compounds in Human LDL. Flavonoid compounds in LDL were determined by using a solid-phase extraction with HPLC−diode array detection (HPLC-DAD) as described in ref *19*. In short, acidulated LDL was applied to a Waters Oasis HLB extraction cartridge and washed with water and 5% aqueous methanol. The flavonoids were eluted with methanol, which was evaporated under a nitrogen stream. The residue was dissolved in glacial acetic acid/water mixture (27:983, v/v), and 20 μL was submitted to HPLC using a LiChrospher RP-18 (250 × 4 mm, 5 μm) column (Merck). The quantitative measurement was performed by integrating the peak area at 280 nm.

Statistical Analysis. Data are analyzed using the Statistical Analysis System software. Significant differences between means are determined by Duncan's multiple-range test.

RESULTS

Effects of Different Concentrations of Glucose on Copper-Mediated Oxidation of LDL. Initially, we attempted to investigate the effect of glucose on copper-mediated LDL oxidation. LDL was incubated with normal (5 mM) or pathophysiologic (15, 30 mM) concentrations of glucose in the presence and absence of copper (Cu^{2+}, 2.4 μM). The extent of LDL lipid peroxidation was monitored by CD and TBARs formation, whereas protein modification of LDL was demonstrated by the increase of REM and ApoB fragmentation on agarose gels. Addition of glucose to the incubation medium was found to accelerate oxidative modification of LDL in the presence of Cu^{2+}. All measures of lipoprotein oxidation, including CD (**Figure 1a**), TBARS (**Figure 1b**), REM (**Figure 1c**), and Apo B fragmentation (**Figure 1d**), were increased in a glucose concentration-dependent manner ($p < 0.05$). In contrast, incubation with glucose (5, 15, and 30 mM) in the absence of Cu^{2+} did not enhance LDL oxidation (data not shown). Thus, glucose-mediated enhancement appeared to be dependent on a reaction pathway that required transition metal ions. In subsequent experiments, a concentration of 30 mM glucose (HG), resulting in significant oxidative modification of LDL (**Figure 1**), was chosen as the concentration representative of diabetes mellitus.

Effects of HG on α- and γ-Tocopherol Consumption in LDL. Vitamin E is the most important lipid-soluble antioxidant found in LDL and probably the most important defense against oxidative damage caused by ROS (*14*). In this regard, we attempted to investigate the kinetics of α- and γ-tocopherol consumption in LDL during oxidation by HG/Cu^{2+}. As shown in **Figure 2**, endogenous α-tocopherol declined rapidly (by 57% from 9.1 to 3.9 nmol/mg of LDL protein) during the first 15 min. Thus, by 30−60 min, almost all of the α-tocopherol disappeared from the HG/Cu^{2+}-treated LDL. Although the content of endogenous γ-tocopherol remained essentially unchanged ($p > 0.05$) during the initial 15 min, as compared to its baseline (time 0 of incubation), it was essentially undetectable after 90 min (**Figure 2**). Thus, within 90 min, exposure to HG (30 mM) led to complete depletion of α- and γ-tocopherols. The results agreed with the report by Esterbauer et al. (*14*) that vitamin E in LDL is highly sensitive to oxidative conditions and acts as a donor antioxidant.

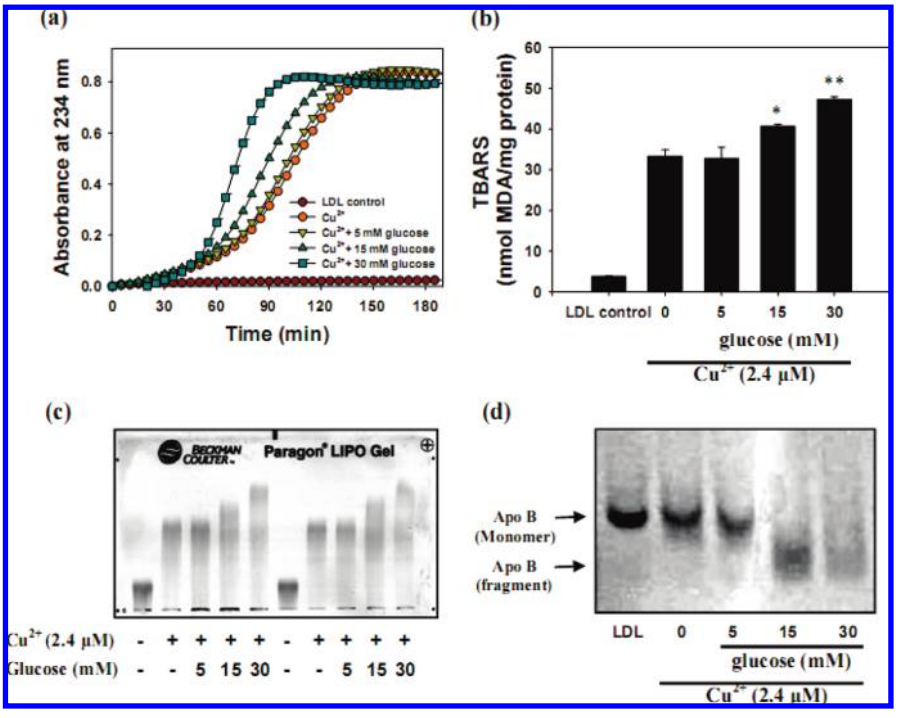

Figure 1. Effects of glucose on copper-mediated LDL oxidation: (**a**) conjugated diene formation [LDL (100 μg of LDL protein/mL) was incubated with different concentrations of glucose (0, 5, 15, and 30 mM) in the presence of 2.4 μM $CuSO_4$; optical density at 234 nm was monitored at 5 min intervals]; (**b**) TBARs formation [LDL samples were measured using a spectrofluorometer (Ex. 515 nm and Em. 553 nm) at 3 h after initiation of oxidation and expressed as nmol of MDA/mg of LDL protein; asterisks indicate significant difference (*, $p < 0.05$; **, $p < 0.01$) from LDL in the absence of glucose]; (**c**) relative electrophoretic mobility of LDL on agarose gels determined at 2 h of incubation; (**d**) ApoB fragmentation [LDL was incubated with the indicated glucose and Cu^{2+} concentrations for 4 h; after incubation, EDTA (final concentration = 1 mM) was added to prevent any further oxidation; SDS-PAGE (3−15% gradient) was carried out with 25 μg of LDL protein' after electrophoresis, each spot was stained with Coomassie Brilliant Blue R250]. Results shown are all representative of three independent experiments.

Effect of LDL-Bound Flavonoids on HG/Cu^{2+}-Mediated LDL Oxidation. To better mimic in vivo conditions, pooled plasma was preincubated with either individual flavonoids or α-tocopherol (as a positive control) at 37 °C for 3 h. LDL was subsequently isolated and its resistance to oxidation tested. CD or TBARs were inhibited by LDL-bound flavonoids, from most to least potent: luteolin, kaempferol, quercetin, naringenin, rutin, EC, catechin, and naringin (**Table 1**). Flavonoid aglycones (quercetin, naringenin) were more effective than their glycosides (rutin, naringin). Flavonoids protected LDL from oxidation, sparing the endogenous α-tocopherol. As summarized in **Table 1**, both LDL-bound flavonoids and α-tocopherol were able to prevent the consumption of endogenous α-tocopherol by 13−75%, relative to the respective baseline (time 0 of incubation = 100%) after 1 h of incubation. At 3 h of incubation or thereafter, LDL-bound catechin and naringin failed to prevent α-tocopherol consumption, whereas LDL-bound luteolin and naringenin led to delayed consumption (70 and 8−16% of α-tocopherol content in LDL preserved at 1 and 3 h, respectively). With regard to α-tocopherol enrichment (positive control), nearly a 2-fold increase in the α-tocopherol content within the LDL particles was observed with similar LDL-bound antioxidant activity, as compared to naringenin enrichment. These results suggested that LDL-bound flavonoids might have the ability to protect against HG-induced α-tocopherol depletion.

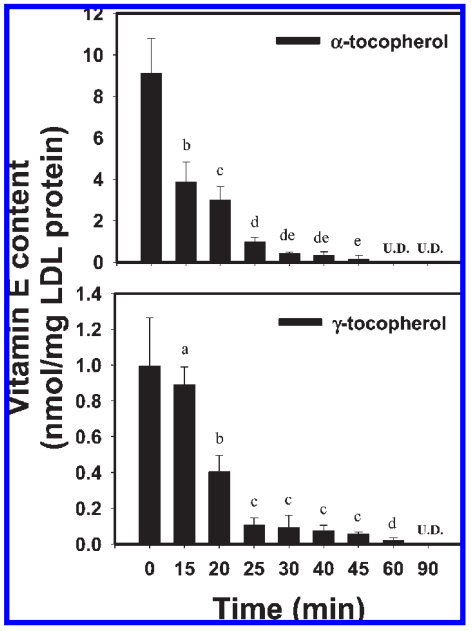

Figure 2. Effects of HG on the consumption of α- and γ-tocopherols in LDL. LDL (100 μg of LDL protein/mL) was incubated in media containing glucose (final concentration = 30 mM). Oxidation was induced with the addition of Cu_2SO_4 (2.4 μM). At different times, aliquots were obtained, and α-tocopherol concentration was determined as described under Materials and Methods. Results shown are representative of the three independent experiments. U.D., undetectable.

Effects of LDL-Bound Flavonoids on HG-Mediated LDL Glycation. Glucose binds nonenzymatically to LDL to form glycated lipoprotein. To explore the possible role of flavonoids in HG-mediated protein glycation, flavonoid-enriched LDL was studied after incubation for 6 weeks with HG without the addition of Cu^{2+}. The results shown in **Table 2** indicated that AGEs-related fluorescence and glycation level (%), as evaluated by boronate affinity chromatography, were significantly higher in LDL after 6 weeks of HG incubation than in native or control LDL ($p < 0.05$). Of all the flavonoids tested, only naringin failed to abrogate LDL glycation. AGEs-related fluorescence assay revealed significant inhibition by rutin, luteolin, and quercetin (38.9, 36.0, and 32.7%, respectively), but not the other flavonoids (2.3−24.3%). LDL-bound flavonoids showed similar variation in their ability to inhibit glycation. Using boronate affinity chromatography, flavonoids were found to suppress the extent of glycation of LDL (glycation %) with the following potency: rutin

Table 1. Effects of LDL-Bound Flavonoids on HG/Cu^{2+}-Mediated Oxidation of LDL[a]

	conjugated dienes[e]		TBARs[f]		α-tocopherol content (nmol/mg of protein)[g] (percentage of 0 h control)		
compound[b]	T_{lag} (min)	ΔT_{lag} (%)	MDA	% IP	0 h	1 h	3 h
native LDL			3.9 ± 0.5h		9.81 (100)	9.97 (102)	9.60 (98)
Cu^{2+} [c]	62.3 ± 4.9g		26.3 ± 1.5cd		8.64 (100)	UD	UD
Cu^{2+}/HG[d]	47.6 ± 2.6h		46.4 ± 1.9a		9.09 (100)	UD	UD
flavanols							
catechin	63.8 ± 6.1g	34.0	34.8 ± 5.6b	12.3	8.87 (100)	2.50 (28)*	UD
EC	75.6 ± 9.5f	58.8	28.7 ± 2.5c	27.7	9.92 (100)	3.37 (34)*	0.16 (2)**
flavanones							
naringenin	112.5 ± 8.9bc	136.9	15.6 ± 3.6ef	60.7	8.10 (100)	5.89 (73)*	0.61 (8)**
naringin	72.2 ± 5.9f	51.7	36.5 ± 5.8b	8.1	9.79 (100)	1.32 (13)	UD
flavonols							
kaempferol	110.1 ± 9.7c	131.3	20.4 ± 1.1d	48.6	8.88 (100)	6.29 (71)*	0.30 (3)**
quercetin	94.2 ± 8.2d	97.9	28.4 ± 3.7c	28.3	9.05 (100)	4.30 (48)*	0.12 (1)**
rutin	82.1 ± 11.3de	72.5	25.8 ± 2.9cd	35.0	9.29 (100)	4.00 (43)*	0.09 (1)**
flavones							
luteolin	127.8 ± 6.2ab	168.5	13.2 ± 1.2f	66.8	9.37 (100)	6.36 (68)*	1.53 (16)**
α-tocopherol	96.8 ± 10.8cd	103.4	27.3 ± 3.2c	31.2	13.20 (100)	3.73 (28)*	UD
DTPA	>600		3.0 ± 0.6h	92.4			
SOD plus catalase	>600		7.7 ± 2.3g	80.6			

[a] Pooled plasma was preincubated with an ethanolic solution of vitamin E and flavonoids to a final concentration of 100 μM, respectively, at 37 °C for 3 h. Native LDL was preincubated with the same amount of pure ethanol. Results are expressed as mean ± SD of triplicate measurements. Values in each column with different letters a−h are significantly different ($p < 0.05$) from each other. [b] DTPA, diethylenetriaminepentaacetic acid (50 μM); SOD, superoxide dismutase (30 μg/mL); catalase (50 μg/mL). [c] Native LDL was incubated with 2.4 μM Cu^{2+} only at 37 °C. [d] Native LDL was incubated with 2.4 μM Cu^{2+} in the presence of 30 mM glucose (HG) at 37 °C. [e] LDL oxidation was monitored by conjugated diene formation at 234 nm every 5 min, and ΔT_{lag} was the difference in lag phase (min) between the Cu^{2+}/HG and flavonoids. T_{lag} % = [(sample T_{lag} − control T_{lag})/control T_{lag}]. [f] The formation of TBARS in the LDL samples was measured using a spectrofluorometer with excitation at 515 nm and emission at 553 nm, expressed as nmol of MDA/mg of LDL protein. The inhibition percentage (IP) was evaluated for TBARS formation by Cu^{2+}/HG-mediated LDL oxidation in each sample. [g] *, **: significantly different from incubation time at 0 h ($p < 0.05$). UD, undetectable.

Table 2. Effects of LDL-Bound Flavonoids on Long-Term Glycation of LDL[a]

compound	AGEs fluorescence (% IP)	glycation (%)
native LDL	33.8 ± 5.5f	2.98 ± 0.60f
NG control	40.1 ± 6.8f	3.23 ± 0.37f
HG control	442.9 ± 37.7a	8.76 ± 0.51a
flavanols		
catechin	372.6 ± 22.8bc (11.9)	7.99 ± 0.28ab
EC	363.6 ± 12.9bc (14.0)	8.03 ± 0.21bc
flavanones		
naringenin	357.5 ± 22.3c (15.5)	7.55 ± 0.26cd
naringin	413.0 ± 35.5ab (2.3)	8.85 ± 0.17a
flavonols		
kaempferol	299.0 ± 13.6d (29.3)	7.10 ± 0.22d
quercetin	284.8 ± 17.1d (32.7)	6.31 ± 0.30e
rutin	258.5 ± 10.2e (38.9)	5.79 ± 0.28e
flavones		
luteolin	270.8 ± 10.7e (36.0)	6.09 ± 0.31e
α-tocopherol	420.8 ± 15.1ab (0.5)	8.52 ± 0.39ab

[a] The extent of LDL glycation was evaluated by measuring the formation of the AGE-related fluorescence and glycation levels (%). HG control and flavonoid-enriched LDL (600 μg/mL) were incubated with glucose (30 mM) at 37 °C for 6 weeks in the dark and under sterile conditions. NG control was incubated with 5.5 mmol/L glucose in the same conditions as HG treatment. Results are expressed as mean ± SD of triplicate measurements. Values with different letters (a−f) are significantly different ($p < 0.05$) from each other.

(33.9%) > luteolin (30.5%) > quercetin (28.0%) > kaempferol (18.9%) > EGCG (17.0%) > naringenin (13.8%) > catechin (8.8%) ≒ EC (8.3%) > naringin (−1.0%). Similar results were obtained by AGEs-related fluorescence assay. Rutin, luteolin, and quercetin dramatically reduced AGEs-related fluorescence and the formation of glycated LDL, whereas reduction in α-tocopherol enrichment was insignificant and did not reduce fluorescence by more than 0.3−0.5%.

Binding Capacities of Flavonoids into LDL. Because plasma lipoproteins have been suggested as potential carriers of flavonoids (*20*), individual flavonoids were preincubated with human plasma followed by density gradient ultracentrifugation and extensive dialysis to isolate LDL and to remove unbound flavonoids. This methodology for LDL incorporation by plasma spiking of pure antioxidants, to better mimic the in vivo conditions, has been reported in several reputable journals (*14*, *19*, *21*−*23*). As shown in **Figure 3**, luteolin (3.3 ± 0.83 μmol/mg of protein), naringenin (2.7 ± 1.08 μmol/mg of protein), kaempferol (2.3 ± 0.85 μmol/mg of protein), and quercetin (2.4 ± 0.98 μmol/mg of protein) were more effective in binding capacity toward LDL among flavonoids tested, indicating that flavonoids were able to bind to LDL, concurrently facilitating the lipoprotein antioxidant (**Table 1**) and antiglycation (**Table 2**) activities against HG. Enrichment of plasma with flavonoids seems to be an efficient means to increase the content of these phenolic compounds in LDL.

DISCUSSION

Poor glycemic control is a common problem for patients with type 1 and type 2 diabetes. It has been suggested that LDL modified by glucose may be more susceptible to oxidation and thus, has enhanced atherogenicity (*5*, *6*). Oxidized LDL (oxLDL) and glycated LDL (glycLDL) have been found to be significantly higher in diabetics than in normal subjects (*3*). Clinical study has also indicated that oxLDL is much higher in patients with impaired glucose tolerance (*24*). First, we found that glucose (5−30 mM) dose dependently promoted copper-induced LDL oxidative modification in vitro, as demonstrated by the formation of CD and TBARs, and enhanced ApoB fragmentation and LDL REM (**Figure 1**). In addition, LDL-associated α- and γ-tocopherol contents were completely consumed by 1−1.5 h during oxidation reactions (**Figure 2**), but this reduction was significantly inhibited by metal chelator (DTPA) or antioxidant enzymes (SOD/catalase) (**Table 1**). Wolff and Dean (*4*) indicated that glucose itself can autoxidize to form hydrogen peroxide in the presence of transition metal ions and subsequently cause lipid peroxidation via a free radical chain reaction mechanism. Some studies also proposed that glucose accelerates LDL oxidation in the presence (*8*) or absence (*7*) of metal ions (Cu^{2+}, Fe^{3+}). It is noteworthy that this pro-oxidant effect can be counteracted by LDL enrichment with vitamin E (*8*). Therefore, it was proposed that the pro-oxidant behavior of HG/Cu^{2+} might be related to (1) site-specific oxidation caused by transition metal ions, (2) ROS generated from the glycation/glycoxidation processes, (3) glucose autoxidation, and (4) the reducing power of glucose itself. The rate and extent of LDL alteration were more marked on reactions with mixtures of glucose and Cu^{2+} than with glucose alone (**Figure 1**).

The main question addressed by the present study was whether enrichment of flavonoids (catechin, EC, kaempferol, luteolin, naringin, naringenin, quercetin, and rutin) into LDL particles inhibited HG-mediated LDL oxidation and glycation. At present, in view of the numerous studies concerning their biofunctional activities, naturally occurring flavonoids have been demonstrated as effective free radical scavengers (*9*−*12*), metal ion chelators (*11*), AGEs inhibitors (*25*), and trapping agents of RCS (*26*). Thus, we hypothesized that flavonoids are effective in slowing LDL oxidation and glycation/glycoxidation in simulated HG conditions of diabetes. Several lines of evidence support this hypothesis. First, we showed that LDL-bound flavonoids, ordered luteolin > naringenin > kaempferol > quercetin > rutin > naringin > EC > catechin, increased the resistance of LDL to HG/Cu^{2+}-mediated oxidation (**Table 1**). Second, introduction of antioxidant flavonoids, such as luteolin, naringenin, or kaempferol, into LDL significantly delayed the consumption of endogenous α-tocopherol under pro-oxidant conditions (**Table 1**). Therefore, it is proposed that LDL-bound flavonoids and LDL-associated α-tocopherol might act as cooperative antioxidants in particles. Third, we also observed that HG-induced glycation of LDL, as evaluated by AGEs-related fluorescence and affinity chromatography, was inhibited by LDL-bound flavonoids, except for naringin (**Table 2**).

Up to now, the protective effects of dietary phenolic compounds on LDL oxidation against metals or pro-oxidant-initiated oxidative modification in vitro have been well observed. However, this should not come as a surprise because experiments with favorable effects for these phenolic agents could be expected, mostly due to their direct chelating action for transition metal ions and/or interaction with oxidants in the reaction medium. Alternatively, phenolic compounds may also be able to protect LDL from oxidative damage by modification of the lipoprotein particles. Although different phenolics have been demonstrated to associate with proteins and membranes through hydrophobic and/or hydrophilic interactions, the data are quite limited and inconsistent. In addition, it is unclear whether these phenolics such as flavonoids bind to the protein or lipid moiety of LDL, and whether their association can provide site-specific protection of LDL components from oxidation and glycation, especially under pathophysiological concentrations of glucose. Notably, a highly possible mechanism by which flavonoid compounds inhibit lipoprotein modifications might be reflected by differences in the affinity of individual flavonoids to lipoproteins as demonstrated by the present study (**Figure 3**).

It is generally accepted that consumption of some plant-derived polyphenols results in their absorption and appearance

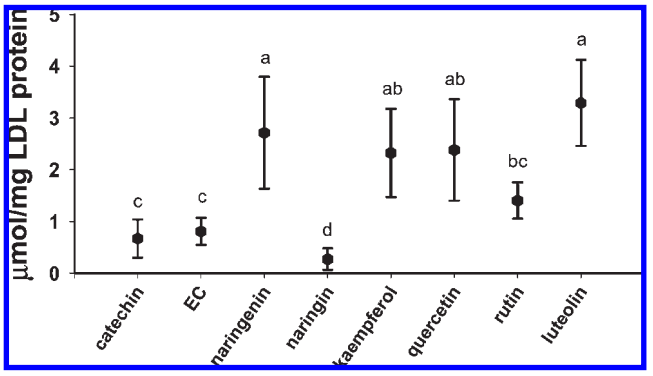

Figure 3. Measurement of flavonoid contents in LDL by introduction of human plasma with individual flavonoid compounds. Results shown are representative of three independent experiments. Groups with different letters (a−d) are significantly different ($p < 0.05$).

in plasma and tissues and protects subjects against cardiovascular diseases (*9*). A few published studies in humans and animals support the antioxidant effects of flavonoids. For example, one study in humans who use soy phytoestrogens as dietary supplements showed that flavonoids do significantly extend the lag time for copper-induced LDL oxidation (*27*). Another study in Apo E deficient mice indicated that isoflavan binds to LDL particles and protects them from oxidation (*28*). Moreover, green tea (*29*), coffee (*30*), red wine or red wine-related polyphenols (*23*, *31*), and olive oil phenols (*21*) attenuate LDL oxidation and prevent α-tocopherol consumption ex vivo and in vivo. Thus, a supplement of flavonoids in response to the inhibition of lipoprotein modification could be a strategy for preventing diabetic complications. Our previous study found that different groups of flavonoids inhibited different stages of protein glycation in vitro (*25*). In the preliminary study of streptozotocin-diabetic rats, flavonoid treatment also decreased AGEs accumulation in multiple tissues and organs (unpublished data). These results suggested that dietary supplementation with flavonoids favorably alters glycotoxin-mediated oxidative and glycoxidative stress in the situation of hyperglycemia, thereby improving their cardiovascular risk profile.

Flavonoids contain both lipophilic and hydrophilic moieties, and therefore they can act against free radicals that are generated in the aqueous and lipid milieu (*11*, *12*). On the basis of structure−activity relationship analysis, we concluded that the C4-carbonyl group was responsible for high antioxidative ability, whereas the C3-hydroxy group might not be necessary. Most flavonoids were ROS scavengers. Miura et al. (*29*) pointed out that sites between the C5-hydroxyl and C4-oxo groups were specialized for chelating metal ions. Consequently, because catechins lack the C4-carbonyl group, they might not inhibit site-specific oxidative damage caused by Cu^{2+} on LDL (*32*). Moreover, lipophilic flavonoids (e.g., aglycones naringenin and quercetin) are more protective than polar flavonoids (e.g., the glycosylated forms, naringin and rutin). These activities run parallel with the degree of lipophilicity (log *P*), which was reported to be naringenin ≒ luteolin > kaempferol ≒ quercetin > rutin > catechin ≒ EC (*33*). Thus, hydrophilic/hydrophobic characteristics of flavonoids might be the key determinant of their activities.

Notably, the antiglycation action of LDL-bound flavonoids was not consistent with their antioxidant capacity in the HG/Cu^{2+} system. It is known that the binding of many antioxidants to LDL is due to the difference in their polarities. Localization of these phytochemicals is the key determinant of their synergistic effects. For example, some hydrophilic compounds, such as lutein and rutin, bind to phospholipids or proteins on the LDL surface, whereas hydrophobic materials, such as β-carotene, bind closer to the LDL core (*22*). Therefore, lipophilic carotenoids have a higher capability than rutin to scavenge lipophilic radicals. This study showed that rutin was the most effective inhibitor of lipoprotein glycation, which contrasted its activity under HG/Cu^{2+} conditions. We speculated that rutin, which is glycosylated, has a greater affinity for the protein domain of lipoproteins (i.e., ApoB), and therefore was better able to inhibit protein glycation of LDL.

In conclusion, HG could accelerate LDL oxidatively via generation of ROS and through glycation/glycoxidation. It was proposed that the binding of individual flavonoids to different sites on the LDL surface protein or internal core is based on their hydrophilic/hydrophobic characteristics. The antioxidant and antiglycation actions of flavonoids might be desirable for the prevention of cardiovascular complications caused by hyperglycemia in diabetic subjects.

ABBREVIATIONS USED

AGEs, advanced glycation endproducts; ApoB, apolipoprotein B; CD, conjugated dienes; EC, epicatechin; HG, high glucose; HPLC-DAD, HPLC−diode array detection; LDL, low-density lipoprotein; NG, normal glucose; REM, relative electrophoretic mobility; RCS, reactive carbonyl species; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBARs, thiobarbituric acid-reactive substances.

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