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Full Length Research Paper

Function-structural study of anti-LDL-oxidation effects of flavonoid phytochemicals

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As a large group of polyphenolic phytochemicals with excellent anti-oxidation properties, the dietary flavonoid intakes have been shown to be negatively correlated with the incidence of coronary artery disease. Flavonoid compounds potentially inhibit the oxidation of low-density lipoprotein (Ox-LDL) that triggers the generation of a series of oxidation byproducts playing important roles in atherosclerosis development. The diverse inhibitory effects of different flavonoid phytochemicals on Ox-LDL might be closely associated with their intrinsic structures. In current work, we investigated the effects of eight flavonoid phytochemicals in high purity (>95%) with similar core structure on the susceptibility of LDL to Cu^{2+}-induced oxidative modification. The results indicated that quercetin, rutin, isoquercitrin, hesperetin, naringenin, hesperidin, naringin and icariin could reduce the Cu^{2+}-induced-LDL oxidation by 59.56±7.03, 46.53±2.09, 40.52±4.65, 22.67±1.68, 20.87±2.43, 12.34±2.09, 10.87±1.68 and 3.53±3.20%, respectively. The function-structure relation study indicated that: (1) for structure similar flavonoids, the flavonoids with more free phenolic hydroxyl groups showed relative higher anti-Ox-LDL activities; (2) the free 3-OH in the C ring of flavones was important for the anti-Ox-LDL activities of flavonol (quercetin) and flavones (rutin and isoquercitrin) as their activities were decreased by ~22 and ~32% following the β-Glc and β-Glc-α-Rha modifications of 3-OH; (3) the 7-OH of A ring was important for anti-Ox-LDL capacities of flavanone compounds. The substitution of β-Glc-α-Rha with the hydrogen of 7-OH made the anti-Ox-LDL abilities for hesperidin and naringin reduced by 45.6 and 47.9%; (4) function and structure comparison also indicated that the C ring C_2-C_3 double bond and the 4'-OH might benefit the protection of Cu^{2+}-induced LDL oxidation by flavonoids. Taken together, the anti-Ox-LDL activities of flavonoids were closely associated with their intrinsic structures/conformations. This study might provide clues to estimate/evaluate the contribution impacts of different functional groups of flavonoids on anti-LDL oxidation, which might also help in selecting effective and favorable dietary flavonoid drugs in the prevention and treatment of coronary artery disease and similar diseases.

Key words: Flavonoid phytochemicals, low-density lipoprotein (LDL) oxidation, antioxidant, function, structure.

INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) plays a pivotal role in atherosclerosis (Wiztum and Steinberg, 1991; Safari and Sheikh, 2003). Oxidation of LDL (Ox-LDL) triggers the generation of a series of oxidation byproducts playing important roles in early development of atherosclerosis (Matsuura et al., 2008). Oxidized LDL (Ox-LDL) is more atherogenic than the native one. The process of Ox-LDL appears to occur within the arterial wall, including endothelial cells, smooth muscle cells and macrophages. The structure alteration

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due to the oxidation allows LDL to be taken up by scavenger receptors on macrophage by promoting the intracellular accumulation of cholesterol, resulting in the formation of lipid-laden foam cells, the hallmark of early atherosclerotic fatty streak lesions (Safari and Sheikh, 2003; Naderi et al., 2003; Aviram and Fuhrman, 2002; Wei et al., 2010; Vaya et al., 2003).

Flavonoids are a class of polyphenol phytochemicals ubiquitously distributed in nature with a broad spectrum of pharmacological properties. Flavonoids are divided into flavonol, flavone, isoflavone, flavanone, flavanol and anthocyanidin (Macready et al., 2009). The polyphenolic flavonoids share a basic 15-carbon skeleton core structure (represented as C_6-C_3-C_6) consisting of two phenylbenzene (chromanol) rings linked through a pyran ring. And their functions are potentially affected by their individual intrinsic structures, the hydroxylation pattern of their molecules as well as the functional groups glycosylated and/or alkylated (Brown et al., 1998; Kostyuk et al., 2003; Cavia-Saiz et al., 2010; Dong and Fan, 2009; Khoo et al., 2010; Rao et al., 2010; Whitman et al., 2005). Considerable epidemiological evidences have revealed that the intake of dietary flavonoid phytochemicals could reduce the incidence and decrease the mortality of coronary artery and myocardial infarction associated diseases (Safari and Sheikh, 2003; Knekt et al., 2002; Dohadwala and Vita, 2009; Kalgaonkar et al., 2010; Mulvihill and Huff, 2010; Arts et al., 2001). Recently, more and more attention have been attracted in the research field of flavonoids as they are showing great potential values in human healthcare (Tuberoso et al., 2009; Heim et al., 2002). Flavonoids show antioxidant properties by scavenging the free radicals, eliminating reactive oxygen species (ROS), chelating transition metal ions and removing potential oxidation initiators (Safari and Sheikh, 2003; Cavia-Saiz et al., 2010; Prochazkova et al., 2011; Beker et al., 2011; Filipe et al., 2001; Zhang et al., 2011; Kyung et al., 2008). They also prevent the destruction of LDL endogenous antioxidants, inhibit cell-mediated oxidation of LDL or enzymes involved in the initiation of oxidation.

Previous researches indicated that the antioxidant activities of flavonoids by scavenging free hydroxyl radicals, peroxyl radicals, eliminating ROS, chelating transition metal ions and removing potential oxidation initiators might be associated with their intrinsic structures/conformations. In this study, we systemically investigated the anti-Cu^{2+}- induced-LDL oxidation activities of eight flavonoid phytochemicals with similar structures, including quercetin, rutin, isoquercitrin, icariin, hesperetin, hesperidin, naringenin and naringin, and explored the relationships between the structures of flavonoids and their anti-Ox-LDL activities. The results indicated minor modifications and/or arrangements of the functional groups of flavonoids apparently affected their anti-Ox-LDL activities. This job provided certain input for estimating/evaluating the contribution impacts of different functional groups of flavonoids for their anti-LDL oxidation. The results from current work might benefit the selection of effective and favorable dietary flavonoid drugs in the prevention and treatment of coronary artery diseases.

MATERIALS AND METHODS

Instruments

The eight flavonoid phytochemicals, quercetin, isoquercitrin, rutin, hesperetin, hesperidin, naringenin, icariin and naringin were generous gifts from Professor Fengxie Jin at the School of Bioengineering, Dalian Polytechnic University, Dalian, China. LDL with purity over 98% was purchased from Guangzhou Yiyuan Biotech. Co. Ltd, China. Evolution 300 UV-Vis spectroscopy was from Thermo Scientific, USA. All other chemicals were analytical grade from commercial sources.

Purity analyses of eight flavonoid phytochemicals by HPLC

0.55 mg of each of the eight flavonoid phytochemicals was dissolved separately in 2 ml of methanol and filtered through a 0.2 µm filter. 10 µl of each flavonoid sample was analyzed by Waters 2690/996 high performance liquid chromatography (HPLC) for purity determination.

An Intersil ODS-3 C18 column (4.6×250 mm) operated at 35°C was used for the HPLC chromatography. The mobile phase for flavonoid samples was 60% (V/V) methanol supplemented with 40% (V/V) of 0.2% phosphoric acid. The elution was performed at a flow rate of 1.0 ml/min. The absorbance wavelength was set at 254 nm for quercetin, isoquercitrin, rutin, hesperetin and hesperidin, 283 nm for naringenin and naringin, and 270 nm for icariin, respectively. Triplicate measurements were performed for each flavonoid sample.

The HPLC chromatographs were collected and processed by using Millennium 32 chromatographic software. HPLC was performed to confirm flavonoid phytochemicals in high purity to ensure all results in positivity.

LDL preparation

The purchased LDL was dialyzed in the dark against 0.01 M phosphate buffered saline (PBS, pH 7.4) overnight to remove ethylenediaminetetraacetic acid (EDTA) at 4°C and adjusted to a protein concentration of 0.05 mg/ml with 0.9% NaCl and filtrated through a 0.22 µm filter, then stored at 4°C before use. Protein concentrations of LDL were determined by the method of Lowry using bovine serum albumin as the standard protein (Weber and Osborn, 1969).

The effect of DMSO on Cu^{2+}-induced LDL oxidation

As we used dimethyl sulfoxide (DMSO) for dissolving the eight flavonoid phytochemicals, we first confirmed whether DMSO would affect the Ox-LDL or not. Briefly, the reaction mixtures (1 ml) containing 970 µl of 0.9% NaCl, 10 µl of 0.05 mg/ml LDL and supplemented with 10 µl DMSO or the control with the same volume of 0.9% NaCl. The oxidation was initiated by the addition of 10 µl of 100 µM $CuSO_4$ at 37°C. Results were presented as the average of three measurements.

Figure 1. The chemical structures of eight flavonoids including quercetin, rutin, isoquercitrin, icariin hesperetin, hesperidin, naringenin and naringin used in the current work.

The inhibitory effect of flavonoids on LDL oxidation

Ox-LDL was performed with a modified method described by Esterbauer et al. (1992). Briefly, the reaction mixtures (1 ml) containing 970 µl of 0.9% NaCl, 10 µl of 0.05 mg/ml LDL and 10 µl of each flavonoid sample dissolved in 10% DMSO or control with the same volume of DMSO were incubated at 37°C for 3 h. The Ox-LDL was initiated by the addition of 10 µl of 100 µM $CuSO_4$ into the aforementioned mixtures at 37°C for 120 min, and then, the oxidation reaction was stopped by the addition of EDTA with the final concentration of 100 µM.

The LDL oxidation were determined by continuously monitoring the absorbance at 234 nm of reaction-produced conjugated dienes (CD) by Evolution 300 UV-Vis spectrophotometer equipped with Peltier temperature control (37°C) and automatically recorded at the interval of 15 min for 120 min.

Data procession and statistical analysis

Data analysis was performed using Statistical Package for Social Sciences (SPSS) 11.5 software. All results are presented as means ± standard deviations (SD) when a minimal number of two independent experiments were performed in triplicate. The differences between groups were evaluated using a one-way analysis of variance (ANOVA) with all pair wise multiple comparison procedures conducted using unpaired *t* test. Values with $P<0.05$ were considered statistically significant differences.

RESULTS

Structural comparisons of eight flavonoid phytochemicals

The eight flavonoid phytochemicals used in the current work were quercetin, isoquercitrin, rutin, hesperetin, hesperidin, naringenin, naringin and icariin that could be cataloged as flavonol (quercetin), flavone (rutin, isoquercitrin and icariin) and flavanone (hesperetin, hesperidin, naringenin and naringin). As the chemical structures schemed in Figure 1, quercetin, rutin, isoquercitrin and icariin are similar chemical derivatives

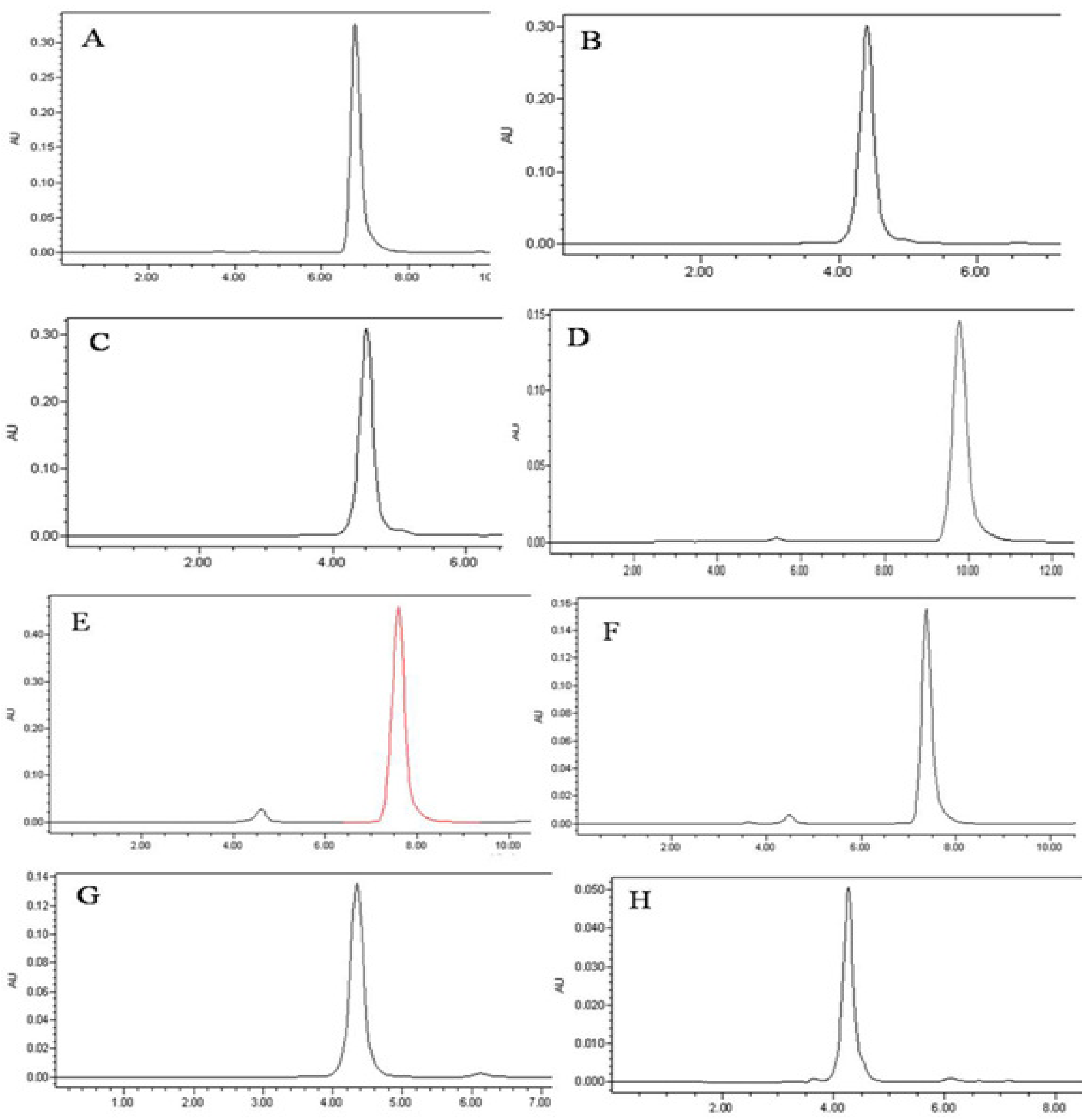

Figure 2. Purity determinations of eight flavonoid phytochemicals by HPLC on an Intersil ODS-3 C18 column (4.6×250 mm). The column was operated at 35°C. The mobile phase was 60% (V/V) methanol supplemented with 40% (V/V) of 0.2% phosphoric acid. Column elution was performed at a flow rate of 1.0 ml/min. The absorbance wavelength was set at 254 nm for quercetin, isoquercitrin, rutin, hesperetin and hesperidin, 283 nm for naringenin and naringin, and 270nm for icariin.

originated from the core structure of flavone, and hesperetin hesperidin, naringenin and naringin share the same core structure of flavanone. The one hand, these flavonoid phytochemicals show similar structure, and on the other hand, they also have their individual intrinsic structures (Figure 1). Therefore, the structure differences among these flavonoid phytochemicals might result in and explain their functional differences.

The eight flavonoid phytochemicals used are in high purity

The HPLC chromatographs of quercetin, rutin, isoquercitrin, icariin, hesperetin, naringenin, hesperidin and naringin were as shown in Figure 2A, B, C, D, E, F, G and H, respectively. The peak of individual flavonoid sample dominated overwhelmingly in its HPLC

Table 1. The purity determinations of eight flavonoid phytochemicals by HPLC.

Flavonoid	N[a]	Wavelength (nm)[b]	Retention time (min)	Purity (area) (%)[c]	Purity (height) (%)[d]
Quercetin	3	254	6.753±0.008	98.22±0.023	98.49±0.018
Isoquercitrin	3	254	4.512±0.012	97.12±0.016	97.20±0.020
Rutin	3	254	4.404±0.009	99.33±0.027	99.33±0.023
Naringenin	3	254	7.379±0.021	95.64±0.017	95.10±0.019
Naringin	3	254	4.261±0.009	95.06±0.036	95.31±0.025
Hesperetin	3	283	7.833±0.015	95.85±0.032	95.02±0.027
Hesperidin	3	283	4.350±0.016	96.53±0.022	96.78±0.034
Icariin	3	270	9.781±0.019	96.85±0.025	96.29±0.028

[a]Represents the number of measurement per experiment; [b]Indicates the wavelength used for monitoring the elution of flavonoid phytochemicals by HPLC; [c]Means the purities of eight flavonoid phytochemicals were calculated based on their peak areas; [d]Represents the purities of eight flavonoid phytochemicals calculated based on their peak heights.

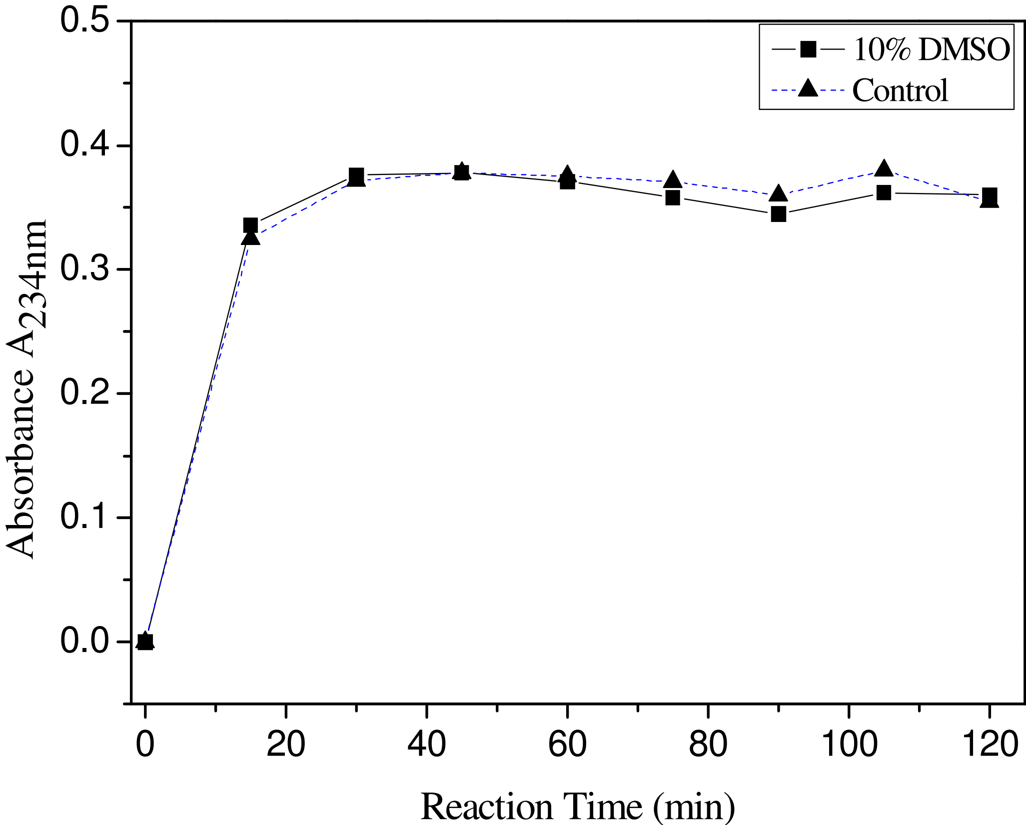

Figure 3. DMSO has no effect on the LDL oxidation induced by Cu^{2+}. 10 µl of 0.05 mg/ml LDL was incubated with 10 µl of 100 µM $CuSO_4$ with and without DMSO (10%) at 37°C. Oxidation was determined by monitoring the change at 234 nm due to increase in CD formation for 120 min. All results are the averages of three measurements.

chromatograph. The purities of eight flavonoid phytochemicals were greater than 95% calculated either based on the HPLC peak area or on the HPLC intensity height (Table 1), which testifies the high purities of flavonoids used for the anti-Ox-LDL experiment, and also ensure that all the results should be obtained with high positivity, accuracy and credibility.

DMSO solvent has no effect on Ox-LDL

As we prepared the eight flavonoid phytochemicals in DMSO, we then examined the effect of DMSO on the oxidation of LDL induced by Cu^{2+}. The formation of CD in the lipid part of LDL gives information about the susceptibility of LDL towards oxidation. Figure 3 showed

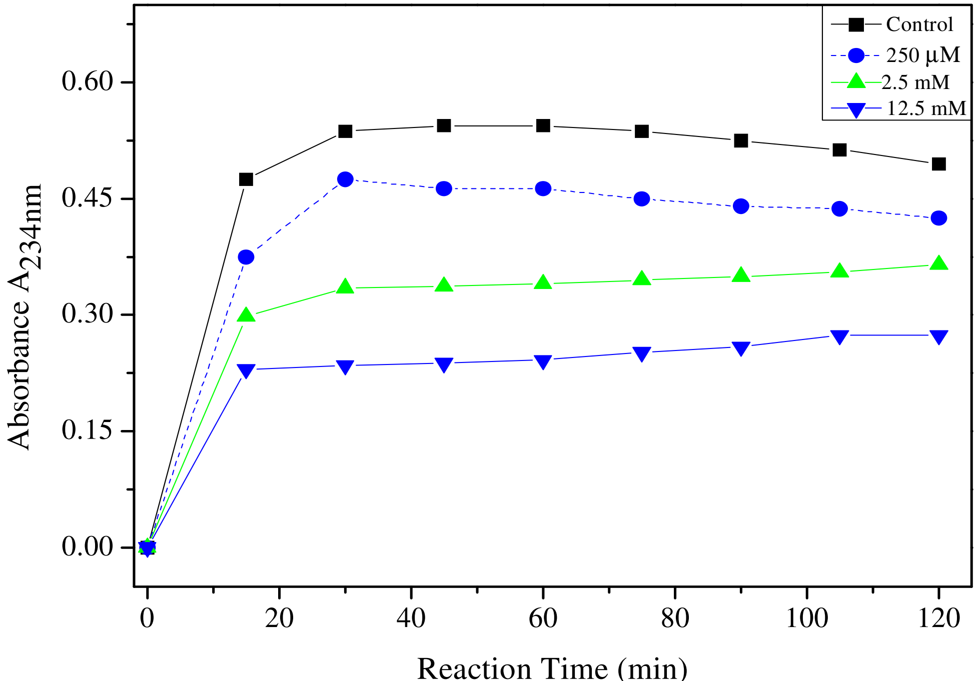

Figure 4. The inhibitory effect of quercetin on Cu^{2+}-induced-LDL oxidation. 10 µl of 0.05 mg/ml LDL was incubated with different concentration of quercetin (250 µM, 2.5 mM and 12.5 mM), oxidation was initiated by the addition of 10 µl of 100 µM $CuSO_4$ at 37°C. Oxidation was determined by monitoring the change at 234 nm due to increase in CD formation for 120 min. All results are the averages of triplicate measurements.

the effect of 10% DMSO on the susceptibility of LDL to Cu^{2+}-induced oxidation as compared to the control. Compare to the control group, the absorbances for CD at 234 nm were pretty comparable and consistent. 10% DMSO used as the solvent for flavonoid phytochemicals in the current work had no effect on the oxidizability of LDL.

Anti-Ox-LDL activities of flavonoid phytochemicals

Quercetin shows anti-Cu^{2+}-induced-Ox-LDL dose-dependently

Figure 4 showed the inhibitory effect of quercetin on LDL oxidation at the concentrations of 250 µM, 2.5 and 12.5 mM. The results indicated that quercetin showed apparent inhibition on the oxidation of LDL induced by Cu^{2+}. Following the increase of quercetin concentration, the absorbance of CD at 234 nm at each time point decreased correspondingly, which indicates that quercetin might show anti-Ox-LDL activity dose-dependently. At the concentration of 12.5 mM, the stable absorbance at 234 nm decreased significantly as compared to the control group quercetin that could protect about 60% of LDL from Cu^{2+}-induced oxidation. We also tried higher concentrations of the eight flavonoid phytochemicals, however, precipitations were observed for half of them when added into the reaction system. Hence, we performed the anti-Ox-LDL activity determinations for the eight flavonoids all at the concentration of 12.5 mM, which also made it possible to compare the relative anti-Ox-LDL activities for all flavonoids at the same level and to explore the structure-function basis. And the anti-Ox-LDL activities of the eight flavonoid phytochemicals was ordered below (according to their inhibitory percentages), quercetin (59.56±7.03%) > rutin (46.53±2.09%) > isoquercitrin (40.52±4.65%) > hesperetin (22.67±1.68%) > naringenin (20.87±2.43%) > hesperidin (12.34± 2.09%) > naringin (10.87±1.68%) > icariin (3.53±3.20%).

Flavonoids with more phenolic hydroxyl groups show relative higher anti- Ox-LDL activity

Comparisons of the structures of eight flavonoid phytochemicals indicated that these compounds can be classified into three types, flavonol, flavone and flavanone (Table 2). The anti-Ox-LDL activity experiment results indicated that inhibitory percentages of Ox-LDL for quercetin, rutin, isoquercetin and icariin were 59.56±7.03, 46.53±2.09, 40.52±4.65 and 3.53±3.20% (Table 2). Quercetin, rutin, isoquercetin and icariin own the same

Table 2. The inhibitory effects of eight flavonoid phytochemicals on LDL oxidation.

Flavonoids subclass	Name of flavonoids	Mw	Inhibitory percentages (%)
Flavone	Rutin (5,7,3',4'-OH)	610.52	46.53±2.09
	Isoquercitrin (5,7, 3',4'-OH)	464.38	40.52±4.65
	Icariin	676.66	3.53±3.20
Flavonol	Quercetin (3,5,7, 3',4'-OH)	302.20	59.56±7.03
Flavanone	Hesperetin(5,7,3'-OH, 4'-OMe)	302.29	22.67±1.68
	Naringenin (5,7,4'-OH)	272.26	20.87±2.43
	Hesperidin (5,3'-OH, 4'-OMe)	610.58	12.34±2.09
	Naringin (5,4'-OH)	580.55	10.87±1.68

core structure of flavones. Overall, the numbers of free phenolic hydroxyl groups for them are 5, 4, 4 and 1. Therefore, the more free phenolic hydroxyl groups a flavone has, the higher anti-Ox-LDL activity it will exhibit. The same situation happens to hesperetin, naringenin, hesperidin and naringin. Owning the same core structure of flavanone, similar conformation, especially both containing four phenolic hydroxyl groups, the hesperetin and naringenin show comparable anti-Ox-LDL activities of 22.67±1.68 and 20.87±2.43% (Table 2). As hesperidin and naringin have similar structures and same number (2) of free phenolic hydroxyl groups, they showed comparable inhibitory percentages of 12.34±2.09 and 10.87±1.68% on Cu^{2+}-induced-Ox-LDL (Table 2).

The 3-OH of C-ring of flavone is important for its anti-Ox-LDL activity

Quercetin, rutin and isoquercitrin share the core structure of flavones (Figure 1). Quercetin showed highest anti-Cu^{2+}-induced-Ox-LDL activity with the inhibitory percentage of 59.56±7.03%, followed by rutin (46.53±2.09%) and isoquercitrin (40.52±4.65%) (Table 2). Compared with quercetin, following the replacements of O-Glc-α-Rha and O-β-Glc of OH at the site of carbon 3 (C_3) of the flavone C-ring (Figure 1), the anti-Ox-LDL activities of rutin and isoquercitrin decreased by ~22 and ~32%, respectively. The aforementioned results indicated that the 3-OH of C-ring of flavone is important for its anti-Ox-LDL activity.

The 7-OH of A-ring of flavanone is important for its anti-Ox-LDL activity

Hesperetin, hesperidin, naringenin and naringin can be regarded as the derivatives originated from same structure core of flavanone (Figure 1). The differences between hesperetin and hesperidin, between naringenin and naringin, are that the 7-OH of A-ring of flavanone is substituted by O-β-Glc-α-Rha. The inhibitory rates of hesperetin, hesperidin, naringenin and naringin on Cu^{2+}-induced-Ox-LDL were 22.67±1.68, 12.34±2.09, 20.87±2.43 and 10.87±1.68%, respectively (Table 2). Obviously, the O-β-Glc-α-Rha substituent of 7-OH decreased the anti-Ox-LDL capacities of hesperetin and naringenin by 45.57 and 47.92%, respectively. The A-ring 7-OH of flavanone is critical for its anti-Ox-LDL function.

C_2-C_3 double bond and 4'-OH might be potentially important for anti-Ox- LDL activity

The quercetin and naringenin decreased the Cu^{2+}-induced-Ox-LDL by 59.56±7.03 and 20.87±2.43%, respectively. Quercetin has C_2-C_3 double bond in C ring, 3-OH and 3',4'-OH, while naringenin has the C_2-C_3 single bond and 4'-OH. Their structures are similar. While they showed significant different anti-Ox-LDL activities, the inhibitory oxidation effect of Ox-LDL was 2.85-fold of that of naringenin. As the 3-OH glycosylation decreased the anti- Ox-LDL activity of quercetin only by ~32%, it can be proposed that the C ring C_2-C_3 double bond and the B

ring 4'-OH might benefit the protection effect of Cu^{2+}-induced LDL oxidation by flavonoids.

DISCUSSION

Flavonoids can react with superoxide anion, hydroxyl radicals and lipid peroxyl radicals, and chelate iron and copper. The antioxidant protection by flavonoids is believed to be caused by a combination of binding to critical sites on LDL, metal chelation and free radical scavenging. Interestingly, dietary flavonoids may contribute to the protection of Ox-LDL for those populations, in particular, whose ascorbic acid (AA) intake are marginal or required to be increased (Safari and Sheikh, 2003; Hetrog et al., 1991; Liu et al., 2004). However, the mechanisms of flavonoids inhibit LDL oxidations still unclear (Cook and Samman, 1996; Kasaoka et al., 2002; Ramos, 2007; Burda and Oleszek, 2001; Pedrielli et al., 2001; Baderschneider and Winterhalter, 2001; Hou et al., 2004).

The phenolic hydroxyl groups of flavonoids are considered to be necessary for their antioxidant activities. It has been reported that flavonoid with more phenolic hydroxyl groups showed stronger antioxidant activity against peroxyl radical (Kostyuk et al., 2003; Cao et al., 1997; Gursoy et al., 2009). Our data also indicated that flavonoids containing more phenolic hydroxyl groups showed relative higher anti-Ox-LDL activities (Table 2). Among all the eight flavonoid phytochemicals tested in the current work, as quercetin contains more phenolic hydroxyl groups (3, 5, 7, 4', 5'-OHs) than the other seven flavonoids, quercetin showed the highest anti-Cu^{2+}-induced-Ox-LDL activity with the inhibitory percentage of 59.56±7.03%. When the 3-O-β-Glc-α-Rha of rutin and 3-O-β-Glc of isoquercitrin were replaced by 3-OH to form quercetin, their anti-Ox-LDL activities were increased by 28.00 and 46.99%, respectively. Rutin and isoquercitrin have no free 3-OH group in the C ring, replaced by the disaccharide glucorhamnoside, which is also the only structural difference from quercetin. This suggests that the 3-OH of C-ring of flavone is important for its anti-Ox-LDL activity. The absence of free 3-OH makes rutin and isoquercitrin not oxidize as readily as quercetin in the presence of Cu^{2+}. The aforementioned results also confirmed that the 3-OH might be the oxidation site of the molecule interacting with Cu^{2+} (Safari and Sheikh, 2003; Naderi et al., 2003; Vaya et al., 2003).

Similar phenomenon was also observed for flavanones, including hesperidin and hesperetin, naringin and naringenin. When the 7-O-β-Glc(6→1)-α-Rha of hesperidin and 7- O-β-Glc(2→1)-α-Rha of naringin were replaced with free 7-OH in the A ring to be hesperetin and naringenin, their Ox-LDL inhibitory abilities were increased from 12.34±2.09 and 10.87±1.68% to 22.67±1.68 and 20.87±2.43% (Table 2), which equivalently increased by 83.71 and 92.00%. Considering the only structural differences between hesperidin and hesperetin and naringin and naringenin were the free 7-OH group replaced by 7-O-β-Glc (6→1)-α-Rha and 7-O-β-Glc(2→1)-α-Rha, the presence of free 7-OH enhances the anti-Ox- LDL activities of flavanones. It might also be implicated that the free 7-OH group in the A ring might be potentially involved in oxidation sites of the molecule interacting with Cu^{2+} (Brown et al., 1998; Tuberoso et al., 2009; Heim et al., 2002). All the aforementioned results indicated clearly that the number and positions of the phenolic hydroxyl groups play important roles for the anti-Ox- LDL activities of flavonoid phytochemicals.

It was reported that the most potential effective flavonoids against oxidation contained two adjacent hydroxyls in the B ring of flavonoid (Yeomans et al., 2005; Zhang et al., 2005). As the 3-OH of rutin and isoquercitrin was glycosylated, comparing to hesperetin and naringenin, the main structural difference of rutin and isoquercitrin is that there are two ortho hydroxyl groups in the B ring, while there is only one hydroxyl group at 3' position of hesperetin and at 4' position of naringenin, which might make their anti-Ox-LDL capacities low. These results also confirmed the importance of the catechol arrangement in the B ring structure (Yeomans et al., 2005; Zhang et al., 2005) for the anti-oxidization activity.

The structure comparison of quercetin and naringenin indicated that the double bond between C_2-C_3 in the C ring, 3-OH and 3',4'-OH of quercetin were replaced by C_2-C_3 single bond and 4'-OH, while the anti-Ox-LDL activity of quercetin was almost three times (2.85-fold) of that of naringenin. As the 3-OH glycosylation only decreased the anti-Ox-LDL activity of quercetin by ~32%, the C_2-C_3 double bond in the C ring as well as the 4'-OH in the B ring might play important role in protecting Cu^{2+}-induced LDL oxidation. The aforementioned results fit well with previous experiment evidences obtained by using other reaction systems (Tuberoso et al., 2009; Heim et al., 2002; Cao et al., 1997).

Taken together, the current work indicated that the anti-Ox-LDL functions of flavonoid phytochemicals are structural related. The anti-Ox-LDL activities of flavonoids were closely associated with their characterized intrinsic structures/conformations. For the flavonoids that share the same structural core, their anti-Ox-LDL activities were also associated with their individual intrinsic structure. More free hydroxyl groups a flavonoid has, the higher anti- Cu^{2+}-induced-Ox-LDL activity it exhibits. The 3-OH of C-ring of flavonoids is important for the anti-Cu^{2+}-induced-Ox-LDL activity. This job also raveled that the C_2-C_3 double bond of C ring and 4'-OH may be potentially associated with the anti-Ox-LDL activity of flavonoids. The presences of two adjacent 3',4'-OH groups at B ring and/or 3-OH (when C ring contains the C_2-C_3 double and a 4'-carboxyl group) might inhibit the Ox-LDL to an appropriate extent. In addition, the 7-OH of A-ring is important for the anti-Cu^{2+}-induced-Ox-LDL activity.

This study might provide certain insights for estimating/evaluating the contribution impacts of different functional groups of flavonoids on the inhibition of LDL oxidation, which might also help in selecting and confirming the effective and favorable dietary flavonoid drugs in the prevention and treatment of coronary artery disease.

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