Differential inhibition of oxidized LDL-induced apoptosis in human endothelial cells treated with different flavonoids

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High plasma level of cholesterol is a well-known risk factor for atherosclerotic diseases. Oxidized LDL induces cellular and nuclear damage that leads to apoptotic cell death. We tested the hypothesis that flavonoids may function as antioxidants with regard to LDL incubated with 5 μM-Cu²⁺ alone or in combination with human umbilical vein endothelial cells (HUVEC). Cytotoxicity and formation of thiobarbituric acid-reactive substances induced by Cu²⁺-oxidized LDL were examined in the presence of various subtypes of flavonoid. Flavanols, flavonols and flavanones at a non-toxic dose of 50 μM markedly inhibited LDL oxidation by inhibiting the formation of peroxidative products. In contrast, the flavones luteolin and apigenin had no such effect, with HUVEC nuclear condensation and fragmentation induced by Cu²⁺-oxidized LDL. In addition, immunochemical staining and Western blot analysis revealed that anti-apoptotic Bcl-2 expression was enhanced following treatment with these protective flavonoids. However, Bax expression and caspase-3 cleavage stimulated by 18h incubation with oxidized LDL were reduced following treatment with these protective flavonoids. The down-regulation of Bcl-2 and up-regulation of caspase-3 activation were reversed by the cytoprotective flavonoids, (−)-epigallocatechin gallate, quercetin, rutin and hesperetin, inhibited HUVEC nuclear condensation and fragmentation induced by Cu²⁺-oxidized LDL. The results suggest that flavonoids may differentially prevent Cu²⁺-oxidized LDL-induced apoptosis and promote cell survival as potent antioxidants. Survival potentials of certain flavonoids against cytotoxic oxidized LDL appeared to stem from their disparate chemical structure. Furthermore, dietary flavonoids may have therapeutic potential for protecting the endothelium from oxidative stress and oxidized LDL-triggered atherogenesis.

Flavonoids: Endothelial apoptosis: Oxidized LDL: Lipid peroxidation: Caspase-3

LDL is oxidized in the sub-endothelial space of the arterial wall and the oxidatively modified LDL is causally involved in human atherosclerosis despite high plasma cholesterol level (Steinberg et al. 1989; Salonen et al. 1992; Ross, 1993). LDL oxidation results in lipid accumulation, focal necrosis covered by smooth muscle cells and surrounded by macrophages, connective tissue proliferation due to chronic inflammation, and other sub-parenchymal events that promote the atherosclerotic process (Schaffner et al. 1980; Gerrity, 1981). Minimally oxidized LDL induces tissue factor expression in cultured human endothelial cells, indicating that this form of modified LDL may be a local mediator promoting thrombosis in atherosclerotic lesions (Drake et al. 1991). Vascular cells exhibit apoptosis in culture upon treatment with oxidized LDL (Colles et al. 2001; Martinet & Kockx, 2001), as do vascular cells of atherosclerotic plaque. It has been recognized that the cytotoxic component of oxidized LDL is one or more oxyresorbs, i.e. 25-hydroxycholesterol and 7-ketocholesterol (Schroepfer, 2000; Colles et al. 2001; Panini & Sinensky, 2001), which may induce apoptosis in the submicromolar range through the mitochondrial pathway (Yang & Sinensky, 2000).

There is growing evidence that an increased intake of phytochemicals such as flavonoids, proanthocyanidins and phenolic acids may be beneficial for cardiovascular health (Geleijnse et al. 2002; Kris-Etherton & Keen, 2002; Blakesmith et al. 2003). It has been shown that wine flavonoids protect against atherosclerosis by inhibiting the accumulation of oxidized LDL in atherogenic lesions of Apo E-deficient mice (Aviram & Fuhrman, 2001; Sartor et al. 2002; Sartor et al. 2002; Sartor et al. 2002). This observation implies that flavonoids confer protection against several events in atherogenic lesion formation, and this phenomenon appears to be associated with their antioxidant capacity (Xu et al. 2004). Flavonoids are natural antioxidants that scavenge various types of radical in aqueous and organic environments (Rice-Evans et al. 1996; Dugas et al. 2000; Xu et al. 2004) and anti-inflammatory agents that inhibit adhesion molecules and matrix proteases (Bito et al. 2002; Sartor et al. 2002; Sartor et al. 2002).

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl (radical); HUVEC, human umbilical vein endothelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; TBARS, thiobarbituric acid-reactive substances; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline–Tween 20; TUNEL, TdT-mediated dUTP nick end labelling.

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Whether flavonoids act in vivo as antioxidants or anti-inflammatory agents appears to depend on their bioavailability.

To test the hypothesis that antioxidative cytoprotection of flavonoids against cytotoxicity of oxidized LDL that appears to be involved in atherogenic lesion formation stems from their chemical structure, we examined the effects of various flavonoids with different antioxidant capacity, applied in submillimolar doses, on atherosclerotic apoptosis in oxidized LDL-exposed human umbilical vein endothelial cells (HUVEC). Cells were exposed to LDL in the presence of 5 μM-Cu²⁺ for killing 30% within 24 h. By measuring cell viability, nuclear morphology, DNA fragmentation and apoptotic gene protein expression, we assessed the anti-apoptotic efficacy of various flavonoids in the vascular model of apoptosis in atherosclerosis. Four different subclasses of flavonoid were used: flavanols ((−)-epigallocatechin gallate and (−)-catechin); flavonols, quercetin, myricetin and rutin; flavanones (naringenin and hesperetin); flavones (luteolin and apigenin).

Materials and methods

Materials

Fetal bovine serum, trypsin– and penicillin–streptomycin were purchased from BioWhittaker (San Diego, CA, USA). Cell growth supplements, flavonoids (flavanols, (−)-epigallocatechin gallate and (−)-catechin; flavonols, quercetin and myricetin; flavanones, naringenin and hesperetin; flavones, luteolin and apigenin), M199 chemicals and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), as were all other reagents unless specifically stated otherwise. All flavonoids were solubilized in dimethyl sulfoxide; the final culture concentration of dimethyl sulfoxide was ≤0.5%.

Plasma LDL preparation

Human plasma LDL was prepared by discontinuous density gradient ultra-centrifugation as previously described (Basu et al. 1976; Kang et al. 2002). The prepared plasma LDL obtained from human normolipidaemic pooled plasma was dialysed overnight against 0.154 M-NaCl and 0.01% EDTA (pH 7–4) at 4°C and was used within 4 weeks of isolation. Protein concentration of the plasma LDL fraction was determined by the Lowry method (Lowry et al. 1951), and concentrations of triacylglycerol, total cholesterol and phospholipid were measured using diagnostic kits (Asan Pharmaceutical Co., Hwasung, Republic of Korea). The contents of total protein, triacylglycerol, total cholesterol and phospholipid in the prepared LDL fraction were all within the appropriate ranges.

LDL oxidation was confirmed by an electrophoretic mobility test. Aliquots of medium were run on a 0.8% agarose electrophoresis gel in barbital buffer (pH 8.6). The gel was immediately fixed in a 5% trichloracetic acid solution and rinsed in 70% ethanol. Photographs of gel were obtained using Polaroid Type 667 positive/negative film (Polaroid Co., Wayland, MA, USA).

Primary cell culture

HUVEC were isolated using collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA), as described elsewhere (Jaffe et al. 1973; Voyta et al. 1984). Cells were incubated in 25 mM-HEPES-buffered M199 containing 10% fetal bovine serum, 2 mM-glutamine, 100 penicillin U/ml, 100 μg streptomycin/ml and growth supplements (0.75 mg human epidermal growth factor/ml and 75 μg hydrocortisone/ml) at 37°C in a humified atmosphere of 5% CO₂ in air. Cells were sub-cultured at 80–90% confluence. Endothelial cells were identified by their cobblestone morphology and uptake of fluorescently labelled acetylated LDL (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes Inc., Eugene, OR, USA; Voyta et al. 1984).

Induction of apoptosis

The cells were pre-incubated for 30 min with 1–50 μM of each test flavonoid and continuously incubated in media containing 0.1 mg LDL/ml and 5 μM-Cu²⁺. Incubation was continued for 24 h before biochemical and molecular analyses were performed. In LDL incubations, LDL oxidation was terminated by adding 2 μM-EDTA at the end of the 24 h incubation period. Controls with or without cells/LDL/Cu²⁺/flavonoids were incubated under the same condition as those used in the LDL protocols. The LDL electrophoretic mobility data proved that the effects for LDL–Cu²⁺ controls without flavonoids were brought about by oxidative modification of LDL and not by Cu²⁺ per se (data not shown).

Measurements of thiobarbituric acid-reactive substances

Oxidative modification of LDL particles to oxidized LDL can be caused by transition metals such as Cu²⁺ (Retsky & Frei, 1995). To measure lipid peroxidation, thiobarbituric acid-reactive substances (TBARS) were measured according to methods described previously with a minor modification (Burge & Aust, 1978). After cells were incubated with LDL and Cu²⁺ for 24 h in the absence and presence of various concentrations of flavonoids, 2 μM-EDTA was added to inhibit further lipid peroxidation and the incubation medium was then collected. Aliquots (1 ml) were centrifuged briefly to remove cell debris and used in the thiobarbituric acid assay, using absorbance at λ = 535 nm. The TBARS contents are expressed as ng malondialdehyde/ml, a product of lipid peroxidation.

Cell viability

At the end of the incubation period the MTT assay was performed to quantify cellular viability (Denizot & Lang, 1986). HUVEC were incubated in a fresh M199 medium containing 1 mg MTT/ml for 3 h at 37°C. The purple formazan product dissolved in 2-propanol was measured colorimetrically at λ = 570 nm with background subtraction at λ = 690 nm.

Nuclear morphology

Nuclear morphology was examined by fluorescence microscopy with an Olympus BX51 fluorescence microscope (Olympus Optical Co., Tokyo, Japan). After the fixation of HUVEC with ice-cold 4% formaldehyde for 1 h, the nuclear stain Hoechst 33258 was added at a final concentration of 10 μg/ml for 1 h to allow uptake and equilibrium before microscopic observation. Cells containing fragmented or condensed nuclei were considered apoptotic, while those containing diffuse and irregular nuclei were considered necrotic (Wyllie et al. 1984).
In situ cell death detection (TdT-mediated dUTP nick end labelling) assay
DNA strand breaks were detected using the nick-end fluorescein-labelling technique (Gavrieli et al. 1992; Choi et al. 2003). To detect the in situ DNA fragmentation, the TdT-mediated dUTP nick end labelling (TUNEL) assay was performed using a commercially available end-labelling kit (Roche Molecular Biochemicals, Penzberg, Germany). For detection and visualization, anti-fluorescein antibody conjugated with alkaline phosphatase was introduced and a substrate solution for the alkaline phosphatase, nitro blue tetrazolium/bromochloroindolyl phosphate toluidine, was added. Photomicroscopy was performed with the Olympus BX51 fluorescence microscope system.

Immunocytochemistry
After washing endothelial cells thoroughly with Tris-buffered saline (TBS) and fixing with 4 % formaldehyde for 20 min, cells were incubated for 1 h with 10 % normal goat serum in TBS to block any non-specific binding. After washing fixed cells twice with TBS, monoclonal mouse Bcl-2 antibody (1:100 dilution in TBS; BD Transduction Laboratories, San Diego, CA, USA) was added to cells, which were then incubated overnight at 4°C. Cells were washed with TBS and incubated with a cyanine-conjugated goat anti-mouse IgG (1:750 dilution in TBS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) as a secondary antibody. Fluorescent images were obtained by the Olympus BX51 fluorescence microscope system.

Western blot analysis
Whole cell extracts were prepared from HUVEC in a lysis buffer containing 10 g β-mercaptoethanol/l, 1 m-β-glycerophosphate, 0-1 m-Na2VO4, 0-5 m-NaF and protease inhibitor cocktail. Cell lysates containing equal amounts of total protein were fractionated by electrophoresis on 15 % SDS–PAGE gels and transferred onto a nitrocellulose membrane. Non-specific binding was blocked by soaking the membrane in TBS-T buffer (0·5 μl-Tris–HCl (pH 7·5), 1·5 m-NaCl and 1 % Tween 20) containing 5 % non-fat dry milk for 3 h. The membrane was incubated overnight at 4°C with a primary antibody (monoclonal mouse anti-human Bax (1:500 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or polyclonal rabbit anti-human caspase-3 (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA). After three washes with TBS-T, the membrane was then incubated for 1 h with a goat anti-mouse IgG or a goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000 dilution; Jackson ImmunoResearch Laboratories, Inc.). The levels of Bax proteins and caspase-3 protein were determined using Supersignal West Pico chemiluminescence detection reagents (Pierce Biotechnology Inc., Rockford, IL, USA) and Konica X-ray film (Konica Co., Tokyo, Japan). Incubation with polyclonal rabbit β-actin antibody (1:1000 dilution; Santa Cruz Biotechnology Inc.) was also performed for the comparative control.

Statistical analyses
The results are presented as mean (SEM). Statistical analyses were conducted using Statistical Analysis Systems statistical software package version 6·12 (sas Institute Inc., Cary, NC, USA). One-way ANOVA was used to determine effects of LDL plus Cu2+ and individual flavonoids. Differences among treatment groups were analysed with Tukey’s multiple-comparison test and were considered significant at P<0·05.

Results
Inhibition of Cu2+-induced LDL oxidation by flavonoids
All tested flavonoids have previously shown little HUVEC cytotoxicity at concentrations ≤ 50 μM (Choi et al. 2003). Accordingly, all the flavonoids at non-toxic submicromolar concentrations were used for culture experiments with LDL. Cell-free control cultures were performed using 0·1 mg LDL-cholesterol/ml in the presence of 5 μM-Cu2+. The oxidative modification of LDL was strongly stimulated by Cu2+, as evidenced by TBARS production (Fig. 1(A)). The effects of flavonoids at 50 μM on LDL oxidation were compared. All the tested flavonoids significantly decreased the Cu2+-stimulated TBARS production. However, in culture with naringenin and apigenin, Cu2+-induced oxidized LDL formation was observed (Fig. 1(A)).

Fig. 1(B) shows that LDL incubation with HUVEC in the absence of Cu2+ appreciably increased LDL oxidation, demonstrating that spontaneous LDL oxidation occurred during incubation even without a Cu2+ addition. As expected, TBARS production increased approximately by threefold when 5 μM-Cu2+ was added to HUVEC for 24 h. HUVEC promoted Cu2+-induced LDL oxidation, compared with that observed in cell-free systems (Fig. 1(A)), possibly due to Cu2+-induced peroxidation of membrane lipids of added cells and/or promotion of LDL oxidation triggered by the added cells. Except for naringenin, luteolin and apigenin, other tested flavonoids at 50 μM almost completely blocked TBARS production. This implies that these antioxidative flavonoids protect LDL against oxidation, thus stabilizing LDL in its native state. In contrast, LDL oxidation was sustained when LDL was treated with 50 μM-naringenin plus 5 μM-Cu2+, relative to LDL exposed to Cu2+ alone (Fig. 1(B)).

Endothelial cell viability under the influence of oxidized LDL
In the absence of LDL, Cu2+ alone at 5 μM maintained HUVEC viable without an induction of HUVEC lipid peroxidation (Kang et al. 2002). Thus, 24 h incubation with 5 μM-Cu2+ was not cytotoxic per se in the absence of LDL. In addition, the LDL electrophoretic mobility data proved that the effects of LDL–Cu2+ without flavonoids were brought about by oxidative modification of LDL. During 24 h incubations, 0·1 mg LDL-cholesterol/ml in the presence of 5 μM-Cu2+ increased the cytotoxicity with a decrease in cell viability by approximately 30 % (Fig. 2). Cu2+-induced oxidative modification of LDL at a concentration of 0·1 mg LDL-cholesterol/ml decreased cell viability by 70 %; this massive cell death was associated with a fivefold increase in TBARS production formation (unpublished data). Each flavonoid inhibited oxidized LDL-induced cell death differently (Fig. 2). Among all the tested flavonoids, 50 μM flavanoids, flavonols and flavanones reduced the rate of oxidized LDL-induced cell death, whereas the same concentration of luteolin and apigenin did not have such protection. The flavonoid protection was associated with a reduction in lipid peroxidation (Fig. 1(B)). However, culture with naringenin failed to fully protect HUVEC against oxidized LDL-induced death (Fig. 2).
Fig. 1. Effects of flavonoids (50 μM) on lipid peroxidation induced by Cu²⁺-oxidized LDL in cell-free systems (A) and in incubation with human umbilical vein endothelial cells (B). After challenge with Cu²⁺-oxidized LDL medium, thiobarbituric acid-reactive substances (TBARS) were measured and expressed as ng malondialdehyde (MDA)/ml. Values are means with standard error of the mean shown by vertical bars for ten determinations. abcdMean values with unlike superscript letters were significantly different (P < 0.05).

Fig. 2. Viability of human umbilical vein endothelial cells after incubation with LDL in the absence and presence of Cu²⁺. Cells were pre-treated with 50 μM of each flavonoid and exposed to 0.1 mg LDL-cholesterol/ml in the presence of 5 μM-CuSO₄ for 24 h. Values are means with standard error of the mean shown by vertical bars for nine determinations, and are expressed as percentage cell survival relative to untreated control cells (cell viability = 100%). abMean values with unlike superscript letters were significantly different (P < 0.05).
which was consistent with incomplete inhibition of Cu²⁺-stimulated TBARS production (Fig. 1(B)).

**Nuclear condensation and DNA fragmentation**

Fig. 3 depicts the flavonoid protection against nuclear condensation and DNA fragmentation of HUVEC induced by fully pre-oxidized LDL. Oxidized LDL induced cells with fragmented and/or condensed nuclei within 24 h and with non-nucleated cell fragment apoptotic bodies (Fig. 3). In control cells not treated with oxidized LDL, there was no sign of morphological nuclear damage and chromatin condensation. The nuclear morphology of cells exposed to fully pre-oxidized LDL with (−)-epigallocatechin gallate, quercetin, rutin and hesperetin was comparable with that of untreated control cells. In marked contrast, the morphology of cells treated with oxidized LDL in the presence of luteolin or apigenin compared poorly with that of the untreated cells. In cells exposed to oxidized LDL with luteolin or apigenin, nuclear condensation and appearance of apoptotic body-like structures became evident with a marked reduction of cell density (Fig. 3). In Fig. 4, the flavonoid protection against nuclear condensation and DNA fragmentation of HUVEC induced by 0·1 mg LDL-cholesterol/ml in the presence of 5 μM-Cu²⁺ is summarized. Results were similar to those in photomicrographs obtained with fully pre-oxidized LDL in Fig. 3. Apoptotic body-like structures disappeared when LDL plus Cu²⁺-treated cells were treated with (−)-epigallocatechin gallate, quercetin, rutin and hesperetin at 50 μM, whereas no protection against internucleosomal DNA fragmentation was observed in the luteolin- and apigenin-treated cells, indicating further induction in apoptosis.

When the in situ TUNEL technique for assessing DNA damage caused by oxidized LDL was applied, there was the expected lack of staining in the LDL-untreated control cells (Fig. 4). However,
heavy nuclear staining in the oxidized LDL-exposed cells was observed. The nuclear DNA fragmentation in oxidized LDL-exposed cells pre-treated with 50 μM (-)-epigallocatechin gallate, quercetin, rutin and hesperetin disappeared almost completely. The other flavonoids did not reduce but rather intensified the TUNEL staining, especially apigenin (Fig. 4). The results of nuclear condensation (Fig. 3) and of DNA fragmentation (Fig. 4) assays suggested that certain flavonoids but not apigenin inhibited oxidized LDL-induced apoptosis in the vascular endothelium.

**Protein expression of Bcl-2 and Bax and activation of caspase-3**

Immunocytochemical assay was used to compare the effects of flavonoids on the oxidized LDL-induced inhibition of Bcl-2 expression. There was substantial cytoplasmic staining in the LDL-free control cells, while this staining disappeared in cells exposed to LDL in the presence of Cu2⁺, suggesting the down-regulation of Bcl-2 expression (Fig. 5). Treatment of oxidized LDL-exposed cells with (-)-epigallocatechin gallate, quercetin or rutin enhanced the Bcl-2 staining. In marked contrast, apigenin did not increase the expression of Bcl-2 mitigated by oxidized LDL.

Changes in Bax expression of HUVEC by incubation with oxidized LDL were investigated (Fig. 6(A)). There was relatively undetectable weak expression of Bax protein in LDL-untreated cells. The Bax protein was obviously up-regulated in oxidized LDL-injured cells relative to the untreated quiescent cells. When cells were pre-treated with the test flavonoids in the presence of oxidized LDL, Bax expression was markedly down-regulated by (-)-epigallocatechin gallate, quercetin, rutin and hesperetin, but not by the flavone apigenin (Fig. 6(A)). When the cytoprotective flavonoids (-)-epigallocatechin gallate, quercetin and hesperetin were added at concentrations between 1 and 50 μM, Bcl-2 down-regulated by oxidized LDL was boosted, with inhibitory doses being ≈10 μM (Fig. 6(B)).

Western blot analysis showed that treatment with LDL in the presence of Cu²⁺ caused caspase-3 cleavage in HUVEC within 24 h, with a maximum activation at 18 h (Fig. 7(A)), and this effect was inhibited by flavonols, flavonols and flavanones but not by flavones (Fig. 7(B)). Pre-incubation of cells with apigenin did not ameliorate the extent of caspase-3 cleavage in response to the toxic effects of oxidized LDL. Caspase-3 activation stimulated by oxidized LDL was mitigated at ≈10 μM (Fig. 7(C)). The caspase-3 activation was significantly blocked by a treatment with10 μM of certain flavonoids, especially (-)-epigallocatechin gallate, quercetin and hesperetin. Thus, to achieve the full inhibitory effect of flavonoids in endothelial apoptotic models, micromolar doses ≥10 μM were required.

**Discussion**

It has been well established that oxidized LDL promotes the atherosclerotic process through lipid accumulation, focal necrosis, connective tissue proliferation and other sub-endothelial events (Schaffner et al. 1980; Gerrity, 1981). Minimally oxidized LDL may be a local mediator promoting thrombosis in atherosclerotic lesions (Drake et al. 1991). The possible cellular mechanisms for oxidized LDL production comprise the lipoxygenase pathway (Zhu et al. 2003), the generation of superoxide radicals most likely at the level of coenzyme Q or via NADH oxidase (Warnholtz et al. 1999) and the generation of thyl radicals (Graham et al. 1996). It has been shown that thyl-dependent oxidative modification of LDL can be accomplished by superoxide-dependent and -independent mechanisms (Heinecke et al. 1993).

In cell-free and cell-containing systems oxidized LDL can be generated by trace amounts of transition metals such as Cu²⁺ and Fe²⁺, or by inorganic oxidants such as H₂O₂ (Retsky & Frei, 1995). In the present study, LDL was oxidized by culture with 5 μM-Cu²⁺ in the endothelial cell system.

Vascular cells in culture upon treatment with oxidized LDL as well as vascular cells of atherosclerotic plaque exhibit apoptosis (Colles et al. 2001; Martinet & Kockx, 2001). Consistent with previous reports showing apoptotic death processes in various types of cells induced by oxidized LDL (Colles et al. 2001; Panini & Sinensky, 2001; Shatrov & Brune, 2003; Alcouffe et al. 2004), the present study demonstrated that treatment of HUVEC with

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![5 μM-Cu²⁺ plus 0-1 mg LDL/ml](https://www.cambridge.org/core/terms). IP address: 54.70.40.11 on 24 Jan 2020 at 15:53:28, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms.)

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![Cu²⁺ plus LDL](https://www.cambridge.org/core/terms). IP address: 54.70.40.11 on 24 Jan 2020 at 15:53:28, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms.)

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Fig. 5. Protein expression of Bcl-2 in human umbilical vein endothelial cells incubated with 50 μM of selected flavonoids and LDL in the presence of Cu²⁺. After fixation, the Bcl-2 localization was detected by immunocytochemical staining with cyanine-conjugated goat anti-mouse IgG. The photographs are representative of four independent slides. Magnification: × 140.
LDL in the presence of Cu\(^{2+}\) leads to cell death via apoptotic processes. However, it has recently been reported that oxidized LDL inhibits macrophage apoptosis by blocking ceramide generation (Hundal et al. 2003). The oxysterols 25-hydroxycholesterol and 7-ketocholesterol, components of oxidized LDL, may induce apoptosis (Schroepfer, 2000; Yang & Sinensky, 2000; Colles et al. 2001; Panini & Sinensky, 2001) through the mitochondrial pathway (Panini & Sinensky, 2001). Accordingly, oxysterols appear to play a role in atherosclerotic cell injury and apoptosis induced by oxidized LDL (Colles et al. 2001). The present study did not measure the formation of oxysterols in Cu\(^{2+}\)-oxidized LDL. Based on these mechanisms causing LDL to oxidized LDL transitions, antioxidants may inhibit production of oxidized LDL, protect cells from the damaging effects of oxygen radicals, and thereby retard atherosclerosis in vivo.

Several studies have revealed that flavonoids could have a considerable antioxidant capacity in various oxidation systems (Rice-Evans et al. 1996; Pannala et al. 1997; Dugas et al. 2000; Zhu et al. 2003). However, each subgroup of flavonoids exhibits different spontaneous scavenging activity against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in the cell-free system (Choi et al. 2003). It has been proposed that there is a relationship between chemical structure and radical scavenging activity of polyphenolic flavonoids (Rice-Evans et al. 1996; Dugas et al. 2000). We have previously shown different inhibitory effects of flavonoids with DPPH scavenging activity on H\(_2\)O\(_2\)-induced endothelial apoptosis (Choi et al. 2003), indicating that there is a major structural feature responsible for the anti-apoptotic activity against oxygen radicals. Also in the present study flavonoids differentially inhibited oxidized LDL-induced apoptosis.

![Fig. 6. Expression of Bax (A) and Bcl-2 (B) in human umbilical vein endothelial cells (HUVEC) incubated with selected flavonoids and/or LDL in the absence and presence of Cu\(^{2+}\). In (A) 50 \(\mu\)M of each flavonoid and in (B) 1–50 \(\mu\)M (-)-epigallocatechin gallate, quercetin and hesperetin were pre-treated prior to an addition of LDL in the absence and presence of Cu\(^{2+}\). Total HUVEC protein extracts were electrophoresed on SDS–PAGE gels, followed by Western blot analysis with a primary antibody against Bax or Bcl-2. \(\beta\)-Actin protein was used as an internal control. Bands are representative of three independent experiments. The bar graphs represent densitometric results for the expression of Bax and Bcl-2 proteins. Values are means with standard error of the mean shown by vertical bars for three determinations. \(^{abc}\)Mean values with unlike superscript letters were significantly different (\(P\leq0.05\)).](https://doi.org/10.1079/BJN20041397)
Antioxidant functions supported the possibility that certain polyphenolics may have production of peroxidative products when each tested flavonoid genic potential of flavonoids were ascertained by measuring cellular injury and appears to be the common apoptotic mediator, underlying mechanisms for cardio- and cytoprotective actions of (Fig. 1(A)). Cu$^{2+}$ enhanced in the cell-containing systems (Fig. 1(B)). These PAGE and Western blot analysis were performed with total HUVEC protein extracts and with a primary antibody against caspase-3. Values are means with standard error of the mean shown by vertical bars for three determinations. abcdef Mean an internal control. Bands are representative of two or three independent experiments. The bar graphs represent densitometric results for the inhibition of caspase-3 activation by flavonoids at various doses. Values are means with standard error of the mean shown by vertical bars for three determinations. abcdef Mean values with unlike superscript letters were significantly different ($P<0.05$).

Fig. 7. Time course (A) and flavonoid inhibition (B) of caspase-3 activation, and flavonoid dose–response (C) to caspase-3 activation in human umbilical vein endothelial cells (HUVEC). Cells were cultured with LDL and Cu$^{2+}$ for 4 to 24 h (A) and for 18 h (B, C). In (B) 50 $\mu$m of each flavonoid and in (C) 1–50 $\mu$m(-)-epi-gallocatechin gallate, quercetin and hesperetin were pre-treated prior to an addition of LDL in the absence and presence of Cu$^{2+}$. After cell incubations, SDS-PAGE and Western blot analysis were performed with total HUVEC protein extracts and with a primary antibody against caspase-3. $\beta$-Actin protein was used as an internal control. Bands are representative of two or three independent experiments. The bar graphs represent densitometric results for the inhibition of caspase-3 activation by flavonoids at various doses. Values are means with standard error of the mean shown by vertical bars for three determinations. abcdef Mean values with unlike superscript letters were significantly different ($P<0.05$).

cytotoxicity. Except for flavone-type flavonoids, luteolin and apigenin, other flavonoids at non-toxic doses inhibited the apoptosis-like alterations including nuclear condensation and DNA fragmentation in the LDL plus Cu$^{2+}$-treated vascular endothelial cells.

Dietary flavonoids are beneficial for cardiovascular health by reducing the incidence of myocardial infarction (Geleijnse et al. 2002). In addition, plasma LDL-cholesterol concentration and lipid peroxidation are alleviated by dietary intakes of flavonoid lipid peroxidation are alleviated by dietary intakes of flavonoid -donating properties (Rice-Evans et al. 1996). The antioxidant actions appear to be mediated through H$^+$-donating properties (Rice-Evans et al. 1996). The underlying mechanisms for cardio- and cytoprotective actions of flavonoids are still unknown. Oxidative stress contributes to cellular injury and appears to be the common apoptotic mediator, most likely via lipid peroxidation (Buttke & Sandstrom, 1994). In the present study, the antioxidant properties and anti-atherogenic potential of flavonoids were ascertained by measuring production of peroxidative products when each tested flavonoid was added in Cu$^{2+}$ plus LDL-containing and cell-free systems (Fig. 1(A)). Cu$^{2+}$ plus LDL-induced lipid peroxidation was enhanced in the cell-containing systems (Fig. 1(B)). These observations indicate that lipid peroxidation occurred both at the level of the LDL particles (see later) and that of the live HUVEC. This quantitative approach proved that certain flavonoids appear to have the potential to reduce spontaneous LDL oxidation and in turn protect HUVEC against peroxidative toxicity (Fig. 2).

It is not known whether the property of cytoprotective flavonoids to stabilize LDL in the presence of Cu$^{2+}$ in vitro translates into true anti-atherogenicity in vivo. Whether flavonoids act in vivo as antioxidants or anti-inflammatory agents appears to depend on their bioavailability. There are considerable differences among the different types of dietary flavonoid such that the most abundant flavonoids in the diet do not necessarily produce the highest concentration of flavonoids or their metabolites in vivo (Kroon et al. 2004; Manach & Donovan, 2004; Walle, 2004). It has been shown that small intestinal absorption ranges from 0 to 60% of the intake dose and the removal half-life ranges from 2 to 28 h (Gugler et al. 1975; Manach & Donovan, 2004; Walle, 2004). Absorbed flavonoids undergo extensive first-pass metabolism in the small intestine epithelial cells and in the liver (Williamson, 2002). The small intestine appears to be the organ primarily responsible for glucuronidation and to play a role in methylation (Manach & Donovan, 2004). Consequently, flavonoid metabolites conjugated with methyl, glucuronate and
sulfate groups are the predominant forms present in plasma. These conjugates are chemically distinct from their parent flavonoids, differing in size, polarity and ionic form, and most likely their physiologic actions. These details should be considered in the design and interpretation of in vitro studies to assess the possible contribution of flavonoids to the potential health effects and to define mechanisms.

It has been reported that flavonoids have metal ion-chelating properties (Milde et al., 2004), in particular true for flavonoids containing a catechol arrangement. Accordingly, Cu\(^{2+}\) binding to LDL for oxidation may vary, depending on individual structural features of flavonoids. Inhibition of Cu\(^{2+}\)-induced LDL oxidation by cytoprotective flavonoids could be due to decreased Cu\(^{2+}\) binding to LDL, a mechanism that appears to be effective with l-ascorbic acid protection (Retsky et al. 1999). Therefore, a several-fold excess of flavonoids over Cu\(^{2+}\) could abolish any effect produced by the LDL–Cu\(^{2+}\) system in controls without flavonoids. In this regard, the direct cytotoxic effect of apigenin in the LDL–Cu\(^{2+}\) system is one that we cannot fully clarify based on our current data. Flavonoids have been discussed to directly inhibit catalytic activities of cell-surface enzymes such as NADH oxidase, cyclooxygenase and cytochrome c oxidase in the systems that are involved in the initiation or propagation of peroxidative products/products. However, the exact mechanism(s) of the positive effect of cytoprotective flavonoids is not known. In addition, since certain flavonoids markedly attenuated the cytotoxicity of oxidized LDL towards HUVEC, it may be likely that the mechanism of flavonoid protection of HUVEC plus LDL was at least in part related to cellular antioxidant metabolism. The current study did not elucidate effects of micro-molar flavonoids on antioxidative defenses such as increased GSH/GSSG ratio.

Certain flavonoids with a potent anti-apoptotic action and flavones without an anti-apoptotic action were tested further for their effects on cascade events of the apoptotic death pathway. The role of reactive oxygen species in oxidized LDL-mediated cytotoxicity has been recently reported, in particular through the activation of the caspase cascade and apoptosis (Hsieh et al., 2001; Vacaresse et al., 2001). As shown in the present study, oxidized LDL promotes the overexpression of Bax and reduces the expression of Bcl-2 or bcl-XL (Salvayre et al., 2002), thereby promoting susceptibility to apoptosis (Kataoka et al., 2001). Bcl-2 expressing cells have been reported to have the enhanced antioxidative capacity that suppresses oxidative stress signals (Voehringer & Meyn, 2000). The oxidized LDL-induced decrease in Bcl-2 protein expression and the increase in Bax expression were blocked in catechin-, flavonol- or flavanone-treated cells, providing compelling evidence in support of their potent anti-apoptotic actions. Oxidized LDL elicited activation of mitochondrial apoptotic and possibly death Fas/FasL receptor pathways.

Oxidized LDL and oxysterols induce the activation of the executioner caspase-3 (Napoli et al., 2000; Wintergerst et al., 2000; Salvayre et al., 2002), via the mitochondrial apoptotic pathway. In addition, caspase inhibitors reduce oxidized LDL-mediated apoptosis (Wintergerst et al., 2000). Nevertheless, the mechanisms linking the oxidized LDL-induced caspase activation are not known. The activated caspase-3 was differentially inhibited by treatment with cathechins, flavonoids and flavanones. The substantial difference between these flavonoids in inhibiting the activated caspase-3 appeared to be responsible for the difference in their anti-apoptotic activities. The cytoprotective flavonoids may switch off the apoptotic death cascade by inhibiting the activation of caspase-3 and likely by boosting the intrinsic cellular tolerance against apoptotic triggers. In contrast, the oxidized LDL-activated caspase-3 was sustained in flavone-treated HUVEC. Thus, our observations suggest that certain flavonoids with anti-apoptotic features may act through endothelial death signalling cascades. Since oxidized LDL activates protein kinases, such as tyrosine kinases and protein kinase C, and mitogen-activated protein kinase pathways (Li et al., 1998; Napoli et al., 2000; Salvayre et al., 2002), it can be speculated that phytochemicals affect multiple signalling pathways that converge at the level of transcriptional regulation (Friso et al., 2002).

In summary, oxidized LDL elicited activation of mitochondrial apoptotic pathways, thereby activating the classical caspase cascade and subsequent apoptotic biochemical and morphological features. Although both oxidized LDL and apoptotic cells are present in atherosclerotic areas, a direct link between oxidized LDL and apoptosis remains to be demonstrated in vivo. Certain flavonoids boosted survival potential against cytotoxic oxidized LDL, which appeared to stem from their disparate chemical structures. Unlike flavones, the flavanols, flavonols and flavanones protected the endothelium from oxidized LDL-mediated apoptotic cell death possibly via mechanisms linked to Bax blockade and anti-apoptotic Bcl-2 expression. It is crucial to elucidate the precise sites of action of anti-apoptotic flavonoids in the sequence of events that regulate oxidant-induced cell death, and to further evaluate the potential of dietary flavonoids as cardio- and cytoprotective agents.

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References


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