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 $5\,\mu M$-Cu^{2+} alone or in combination with human umbilical vein endothelial cells (HUVEC). Cytotoxicity and formation of thiobarbituric acid-reactive substances induced by Cu^{2+}-oxidized LDL were examined in the presence of various subtypes of flavonoid. Flavanols, flavonols and flavanones at a non-toxic dose of $50\,\mu M$ markedly inhibited LDL oxidation by inhibiting the formation of peroxidative products. In contrast, the flavones luteolin and apigenin had no such effect, with $>30\%$ of cells killed after exposure to 0·1 mg LDL/ml. Protective flavonoids, especially $(-)$-epigallocatechin gallate, quercetin, rutin and hesperetin, inhibited HUVEC nuclear condensation and fragmentation induced by Cu^{2+}-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 18 h 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 and hesperetin, at $\geq 10\,\mu M$. These results suggest that flavonoids may differentially prevent Cu^{2+}-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 subparenchymal 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 oxysterols, 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 atherosclerotic lesions, paraoxonase elevation and removal of atherogenic lesions of Apo E-deficient mice (Aviram & Fuhrman, 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). 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 $5 \mu M$-Cu^{2+} 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 % trichloroacetic 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 humidified atmosphere of 5 % CO_2 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′,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 $1–50 \mu M$ of each test flavonoid and continuously incubated in media containing 0·1 mg LDL/ml and $5 \mu M$-Cu^{2+}. Incubation was continued for 24 h before biochemical and molecular analyses were performed. In LDL incubations, LDL oxidation was terminated by adding $2 \mu M$-EDTA at the end of the 24 h incubation period. Controls with or without cells/LDL/Cu^{2+}/flavonoids were incubated under the same condition as those used in the LDL protocols. The LDL electorophoretic mobility data proved that the effects for LDL–Cu^{2+} controls without flavonoids were brought about by oxidative modification of LDL and not by Cu^{2+} *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 Cu^{2+} (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 Cu^{2+} for 24 h in the absence and presence of various concentrations of flavonoids, $2 \mu M$-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 $\lambda = 535$ 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 $\lambda = 570$ nm with background subtraction at $\lambda = 690$ 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 33 258 was added at a final concentration of 10 µg/ml for 1 h to allow uptake and equilibration 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-Na_3VO_4, 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 M-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 Cu^{2+} 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 Cu^{2+}-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-Cu^{2+}. The oxidative modification of LDL was strongly stimulated by Cu^{2+}, 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 Cu^{2+}-stimulated TBARS production. However, in culture with naringenin and apigenin, Cu^{2+}-induced oxidized LDL formation was observed (Fig. 1(A)).

Fig. 1(B) shows that LDL incubation with HUVEC in the absence of Cu^{2+} appreciably increased LDL oxidation, demonstrating that spontaneous LDL oxidation occurred during incubation even without a Cu^{2+} addition. As expected, TBARS production increased approximately by threefold when 5 μM-Cu^{2+} was added to HUVEC for 24 h. HUVEC promoted Cu^{2+}-induced LDL oxidation, compared with that observed in cell-free systems (Fig. 1(A)), possibly due to Cu^{2+}-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-Cu^{2+}, relative to LDL exposed to Cu^{2+} alone (Fig. 1(B)).

Endothelial cell viability under the influence of oxidized LDL

In the absence of LDL, Cu^{2+} 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-Cu^{2+} was not cytotoxic *per se* in the absence of LDL. In addition, the LDL electrophoretic mobility data proved that the effects of LDL–Cu^{2+} 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-Cu^{2+} increased the cytotoxicity with a decrease in cell viability by approximately 30 % (Fig. 2). Cu^{2+}-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 flavanols, 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),
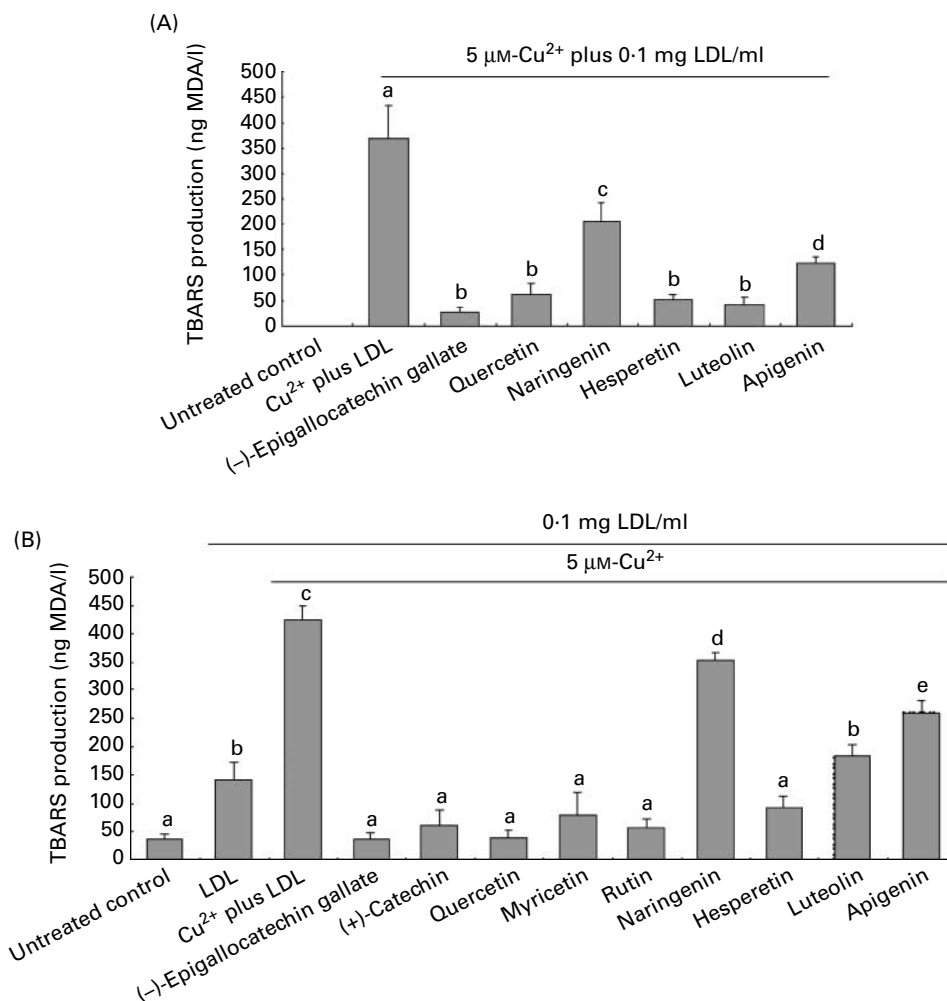
(A)

(B)

Fig. 1. Effects of flavonoids (50 μM) on lipid peroxidation induced by Cu^{2+}-oxidized LDL in cell-free systems (A) and in incubation with human umbilical vein endothelial cells (B). After challenge with Cu^{2+}-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. [abcd]Mean values with unlike superscript letters were significantly different ($P<0.05$).
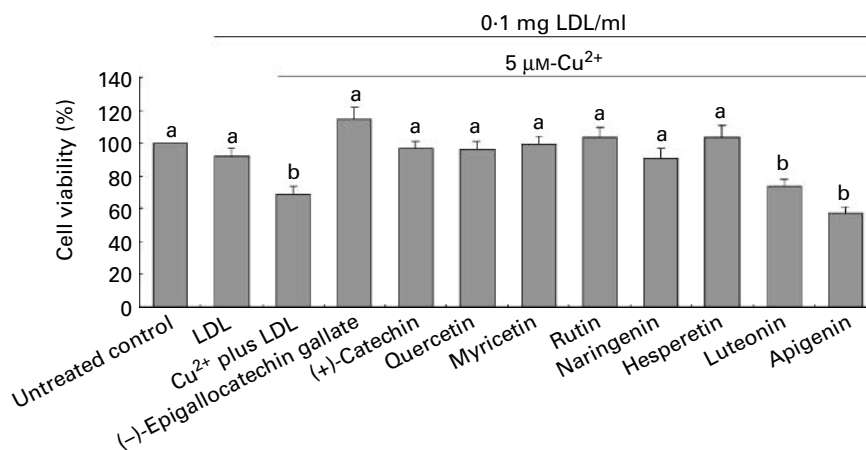

Fig. 2. Viability of human umbilical vein endothelial cells after incubation with LDL in the absence and presence of Cu^{2+}. Cells were pre-treated with 50 μM of each flavonoid and exposed to 0·1 mg LDL-cholesterol/ml in the presence of 5 μM-CuSO$_4$ for 24 h. Values are means with standard error of the mean shown by vertical bars for nine determinations, and are expressed as percentage cell survival relative to untreated control cells (cell viability = 100 %). [ab]Mean values with unlike superscript letters were significantly different ($P<0.05$).
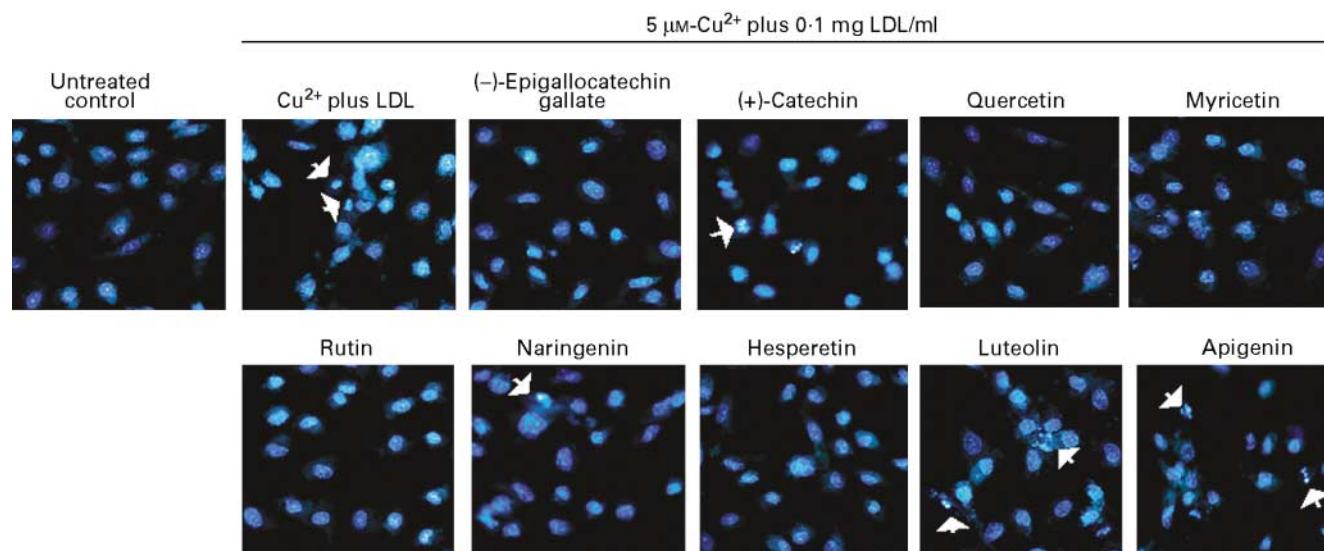
5 μM-Cu²⁺ plus 0·1 mg LDL/ml

Fig. 3. Nuclear morphology of human umbilical vein endothelial cells (HUVEC) pre-treated with 50 μM of each flavonoid and exposed to 0·1 mg LDL-cholesterol/ml plus 5 μM-CuSO₄. HUVEC nuclear morphology was stained with Hoechst 33 258 (Sigma Chemical Co., St Louis, MO, USA). →, apoptosis-like bodies with nuclear condensation. The photomicrographs are representative of four independent slides. Magnification: × 140.

which was consistent with incomplete inhibition of Cu^{2+}-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 $5 \mu M$-Cu^{2+} 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 Cu^{2+}-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,
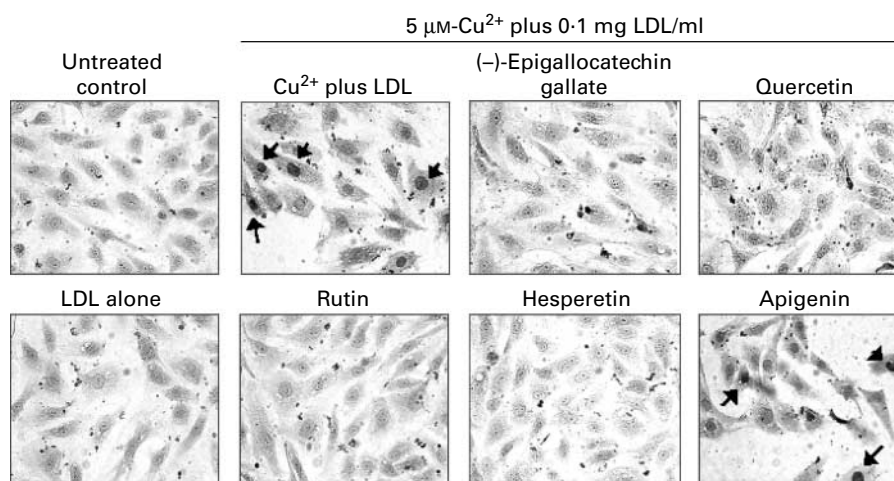
5 μM-Cu²⁺ plus 0·1 mg LDL/ml

Fig. 4. *In situ* DNA fragmentation of human umbilical vein endothelial cells (HUVEC) pre-treated with 50 μM of each flavonoid and exposed to 0·1 mg LDL-cholesterol/ml plus 5 μM-CuSO₄. DNA strand breaks in HUVEC were stained by TdT-mediated dUTP nick end labelling. →, heavy nuclear staining in the oxidized LDL-exposed cells. The photomicrographs are representative of five independent slides. Magnification: × 140.

heavy nuclear staining in the oxidized LDL-exposed cells was observed. The nuclear DNA fragmentation in oxidized LDL-exposed cells pre-treated with $50\,\mu\text{M}$-$(-)$-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 Cu^{2+}, 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 $50\,\mu\text{M}$, Bcl-2 down-regulated by oxidized LDL was boosted, with inhibitory doses being $\geq 10\,\mu\text{M}$ (Fig. 6(B)).

Western blot analysis showed that treatment with LDL in the presence of Cu^{2+} 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 flavanols, 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 $\geq 10\,\mu\text{M}$ (Fig. 7(C)). The caspase-3 activation was significantly blocked by a treatment with $10\,\mu\text{M}$ of certain flavonoids, especially $(-)$-epigallocatechin gallate, quercetin and hesperetin. Thus, to achieve the full inhibitory effect of flavonoids in endothelial apoptotic models, micromolar doses $\geq 10\,\mu\text{M}$ 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-parenchymal 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 thiyl radicals (Graham *et al.* 1996). It has been shown that thiol-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 Cu^{2+} and Fe^{2+}, or by inorganic oxidants such as H_2O_2 (Retsky & Frei, 1995). In the present study, LDL was oxidized by culture with $5\,\mu\text{M}$-Cu^{2+} 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
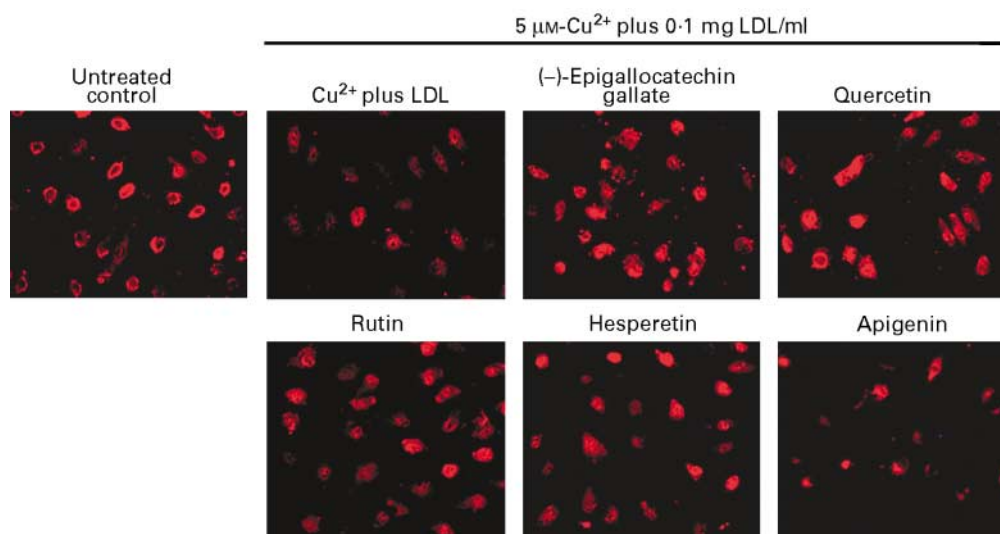

Fig. 5. Protein expression of Bcl-2 in human umbilical vein endothelial cells incubated with $50\,\mu\text{M}$ of selected flavonoids and LDL in the presence of Cu^{2+}. After fixation, the Bcl-2 localization was detected by immunocytochemical staining with cyanine-conjugated goat anti-mouse IgG. The photographs are representative of four independent slides. Magnification: $\times 140$.
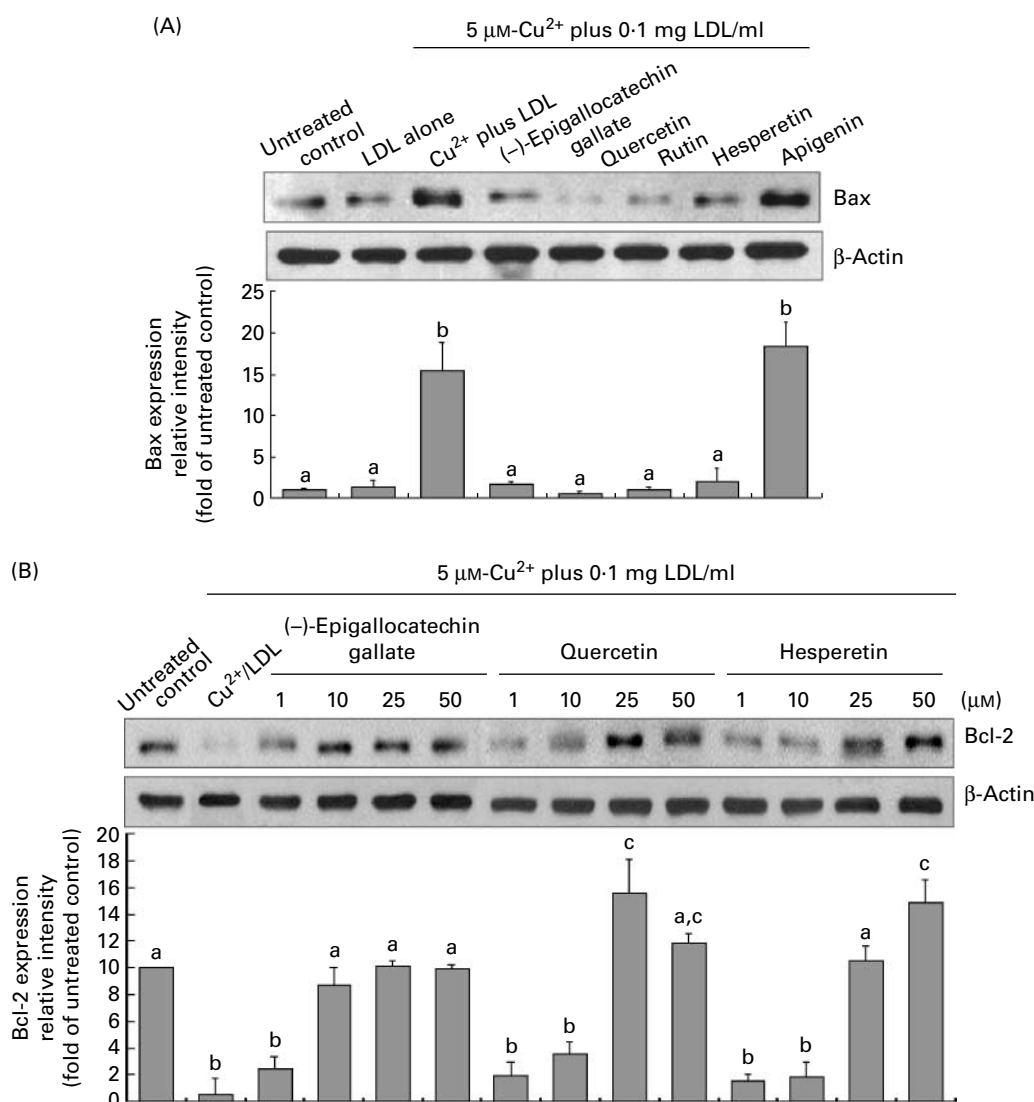
Fig. 6. Expression of Bax (A) and Bcl-2 (B) in human umbilical vein endothelial cells (HUVEC) incubated with selected flavonoids and/or LDL in the absence and presence of Cu^{2+}. In (A) 50 μM of each flavonoid and in (B) 1–50 μM- (−)-epigallocatechin gallate, quercetin and hesperetin were pre-treated 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. β-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 ($P < 0.05$).

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_2O_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
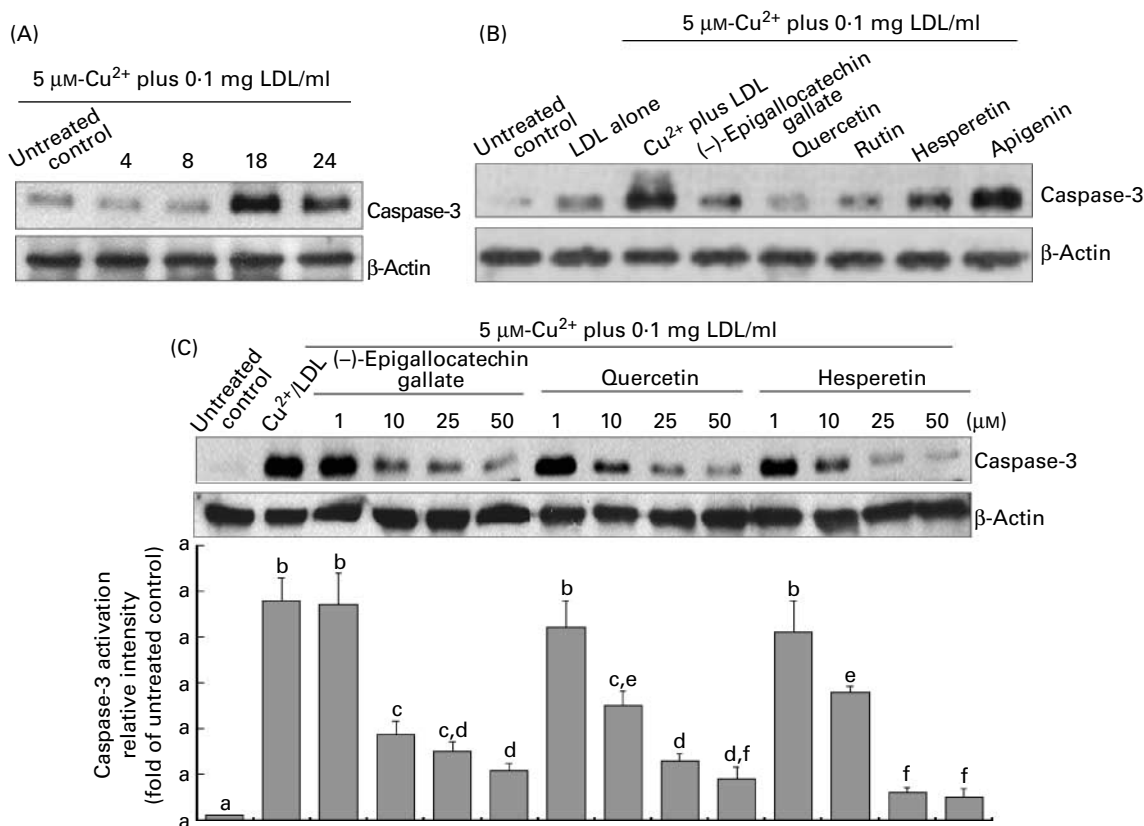
Fig. 7. Time course (A) and flavonoid inhibition (B) of caspase-3 activation, and flavonoid dose–response (C) to caspase-3 activation in human umbilical vein endothelial cells (HUVEC). Cells were cultured with LDL and Cu^{2+} for 4 to 24 h (A) and for 18 h (B, C). In (B) 50 μM of each flavonoid and in (C) 1–50 μM-(−)-epigallocatechin 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. β-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 (Freese *et al.* 1999). Studies exploiting rodent models have supported the possibility that certain polyphenolics may have antioxidant functions *in vivo* (Funabiki *et al.* 1999; Giovannini *et al.* 2001). 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/processes. 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 micromolar flavonoids on antioxidant defences 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 antioxidant 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 catechins, flavonols 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 (Frigo *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

Alcouffe J, Therville N, Segui B, Nazzal D, Blaes N, Salvayre R, Thomsen M & Benoist H (2004) Expression of membrane-bound and soluble FasL in Fas- and FADD-dependent T lymphocyte apoptosis induced by mildly oxidized LDL. *FASEB J* **18**, 122–124.

Aviram M & Fuhrman B (2002) Wine flavonoids protect against LDL oxidation and atherosclerosis. *Ann N Y Acad Sci* **957**, 146–161.

Basu SK, Goldstein JL, Anderson RGW & Brown MS (1976) Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc Natl Acad Sci USA* **73**, 3178–3182.

Bito T, Roy S, Sen CK, Shirakawa T, Gotoh A, Ueda M, Ichihashi M & Packer L (2002) Flavonoids differentially regulate IFN gamma-induced ICAM-1 expression in human keratinocytes: molecular mechanisms of action. *FEBS Lett* **520**, 145–152.

Blakesmith SJ, Lyons-Wall PM, George C, Joannou GE, Petocz P & Samman S (2003) Effects of supplementation with purified red clover (*Trifolium pratense*) isoflavones on plasma lipids and insulin resistance in healthy premenopausal women. *Br J Nutr* **89**, 467–474.

Burge JA & Aust SD (1978) Microsomal lipid peroxidation. *Methods Enzymol* **52**, 302–310.

Buttke TM & Sandstrom PA (1994) Oxidative stress as a mediator of apoptosis. *Immunol Today* **15**, 7–10.

Choi YJ, Kang JS, Park JH, Lee YJ, Choi JS & Kang YH (2003) Polyphenolic flavonoids differ in their antiapoptotic efficacy in hydrogen

peroxide-treated human vascular endothelial cells. *J Nutr* **133**, 985–991.

Colles SM, Maxson JM, Carlson SG & Chisholm GM (2001) Oxidized LDL-induced injury and apoptosis in atherosclerosis. Potential roles for oxysterols. *Trends Cardiovasc Med* **11**, 131–138.

Denizot F & Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modification to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* **89**, 271–277.

Drake TA, Hanani K, Fei HH, Lavi S & Berliner JA (1991) Minimally oxidized low density lipoprotein induces tissue factor expression in cultured human endothelial cells. *Am J Pathol* **138**, 601–607.

Dugas AJ Jr, Castaneda-Acosta J, Bonin GC, Price KL, Fischer NH & Winston GW (2000) Evaluation of the total peroxyl radical-scavenging capacity of flavonoids: structure–activity relationships. *J Nat Prod* **63**, 327–331.

Freese R, Basu S, Hietanen E, Nair J, Nakachi K, Bartsch H & Mutanen M (1999) Green tea extract decreases plasma malondialdehyde concentration but does not affect other indicators of oxidative stress, nitric oxide production, or hemostatic factors during a high-linoleic acid diet in healthy females. *Eur J Nutr* **38**, 149–157.

Frigo DE, Duong BN, Melnik LI, Schief LS, Collins-Burow BM, Pace DK, McLachlan JA & Burow ME (2002) Flavonoid phytochemicals regulate activator protein-1 signal transduction pathways in endometrial and kidney stable cell lines. *J Nutr* **132**, 1848–1853.

Funabiki R, Takeshita K, Miura Y, Shibasato M & Nagasawa T (1999) Dietary supplement of G-rutin reduces oxidative damage in the rodent model. *J Agric Food Chem* **47**, 1078–1082.

Gavrieli Y, Sherman Y & Ben-Sasson SA (1992) Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J Cell Biol* **119**, 493–501.

Geleijnse JM, Launer LJ, Van der Kuip DA, Hofman A & Witteman JC (2002) Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam Study. *Am J Clin Nutr* **75**, 880–886.

Gerrity RG (1981) The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am J Pathol* **103**, 181–190.

Giovannini L, Migliori M, Longoni BM, Das DK, Bertelli AA, Panichi V, Filippi C & Bertelli A (2001) Resveratrol, a polyphenol found in wine, reduces ischemia reperfusion injury in rat kidneys. *J Cardiovasc Pharmacol* **37**, 262–270.

Graham A, Wood JL, O'Leary VJ & Stone D (1996) Human (THP-1) macrophages oxidize LDL by a thiol-dependent mechanism. *Free Radic Res* **25**, 181–192.

Gugler R, Leschik M & Dengler HJ (1975) Disposition of quercetin in man after single oral and intravenous doses. *Eur J Clin Pharmacol* **9**, 229–234.

Heinecke JW, Kawamura M, Suzuki L & Chait A (1993) Oxidation of low density lipoprotein by thiols: superoxide-dependent and -independent mechanisms. *J Lipid Res* **34**, 2051–2061.

Hsieh CC, Yen MH, Yen CH & Lau YT (2001) Oxidized low density lipoprotein induces apoptosis via generation of reactive oxygen species in vascular smooth muscle cells. *Cardiovasc Res* **49**, 135–145.

Hundal RS, Gomez-Munoz A, Kong JY, Salh BS, Marotta A, Duronio V & Steinbrecher UP (2003) Oxidized low density lipoprotein inhibits macrophage apoptosis by blocking ceramide generation, thereby maintaining protein kinase B activation and Bcl-XL levels. *J Biol Chem* **278**, 24399–24408.

Jaffe EA, Nachman RL, Becker CG & Minick CR (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* **52**, 2745–2756.

Kang YH, Park SH, Lee YJ, Kang JS, Kang IJ, Shin HK, Park JHY & Bunger R (2002) Antioxidant α-keto-carboxylate pyruvate protects low-density lipoprotein and atherogenic macrophages. *Free Radic Res* **36**, 905–914.

Kataoka H, Kume N, Miyamoto S, Minami M, Morimoto M, Hayashida K, Hashimoto N & Kita T (2001) Oxidized LDL modulates Bax/

Bcl-2 through the lectin like Ox-LDL receptor-1 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **21**, 955–960.

Kris-Etherton PM & Keen CL (2002) Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. *Curr Opin Lipidol* **13**, 41–49.

Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C & Williamson G (2004) How should we assess the effects of exposure to dietary polyphenols *in vitro*? *Am J Clin Nutr* **80**, 15–21.

Li D, Yang B & Mehta JL (1998) Ox-LDL induces apoptosis in human coronary artery endothelial cells: role of PKC, PTK, bcl-2, and Fas. *Am J Physiol* **275**, H568–H576.

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.

Manach C & Donovan JL (2004) Pharmacokinetics and metabolism of dietary flavonoids in humans. *Free Radic Res* **38**, 771–785.

Martinet W & Kockx MM (2001) Apoptosis in atherosclerosis: focus on oxidized lipids and inflammation. *Curr Opin Lipidol* **12**, 535–541.

Milde J, Elstner EF & Grassmann J (2004) Synergistic inhibition of low-density lipoprotein oxidation by rutin, γ-terpinene, and ascorbic acid. *Phytomedicine* **11**, 105–113.

Napoli C, Quehenberger O, De Nigris F, Abete P, Glass CK & Palinski W (2000) Mildly oxidized low density lipoprotein activates multiple apoptotic signaling pathways in human coronary cells. *FASEB J* **14**, 1996–2007.

Panini SR & Sinensky MS (2001) Mechanisms of oxysterol-induced apoptosis. *Curr Opin Lipidol* **12**, 529–533.

Pannala AS, Rice-Evans CA, Halliwell B & Singh S (1997) Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols. *Biochem Biophys Res Commun* **232**, 164–168.

Retsky KL & Frei B (1995) Vitamin C prevents metal ion-dependent initiation and propagation of lipid peroxidation in human low-density lipoprotein. *Biochim Biophys Acta* **1257**, 279–287.

Retsky KL, Chen K, Zeind J & Frei B (1999) Inhibition of copper-induced LDL oxidation by vitamin C is associated with decreased copper binding to LDL and 2-oxo-histidine formation. *Free Radic Biol Med* **26**, 90–98.

Rice-Evans CA, Miller NJ & Paganga G (1996) Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* **20**, 933–956.

Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**, 801–809.

Salonen JT, Ylä-Hertuala S, Yamamoto R, Butler S, Korpela H, Salonen R, Nyyssonen K, Palinski W & Witztum JL (1992) Autoantibody against oxidised LDL and progression of carotid atherosclerosis. *Lancet* **339**, 883–887.

Salvayre R, Auge N, Benoist H & Negre-Salvayre A (2002) Oxidized low-density lipoprotein-induced apoptosis. *Biochim Biophys Acta* **1585**, 213–221.

Sartor L, Pezzato E, Dell'Aica I, Caniato R, Biggin S & Garbisa S (2002) Inhibition of matrix-proteases by flavonoids: chemical insights for anti-inflammatory and anti-invasion drug design. *Biochem Pharmacol* **64**, 229–237.

Schaffner T, Taylor K, Bartucci EJ, Fischer-Dzoga J, Beeson H, Glagov S & Wissler RW (1980) Arterial foam cells with distinctive immuno-morphologic and histochemical features of macrophages. *Am J Pathol* **100**, 57–80.

Schroepfer GJ Jr (2000) Oxysterols: modulators of cholesterol metabolism and other processes. *Physiol Rev* **80**, 361–554.

Shatrov VA & Brune B (2003) Induced expression of manganese super-oxide dismutase by non-toxic concentrations of oxidized low-density lipoprotein (oxLDL) protects against oxLDL-mediated cytotoxicity. *Biochem J* **374**, 505–511.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC & Witztum JL (1989) Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* **320**, 915–924.

Vacaresse N, Vieira O, Robbesyn F, Jürgens G, Salvayre R & Negre-Salvayre A (2001) Phenolic antioxidants trolox and caffeic acid

modulate the oxidized LDL-induced EGF-receptor activation. *Br J Pharmacol* **132**, 1777–1788.

Voehringer DW & Meyn RE (2000) Redox aspects of bcl-2 function. *Antioxid Redox Signal* **2**, 537–550.

Voyta JC, Via DP, Butterfield CE & Zetter BR (1984) Identification and isolation of endothelial cells based on their increased uptake of acetyl-low density lipoprotein. *J Cell Biol* **99**, 2034–2040.

Walle T (2004) Absorption and metabolism of flavonoids. *Free Radic Biol Med* **36**, 829–837.

Warnholtz A, Nickenig G, Schulz E, et al. (1999) Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation* **99**, 2027–2033.

Williamson G (2002) The use of flavonoid aglycones in *in vitro* systems to test biological activities: based on bioavailability data, is this a valid approach? *Phytochem Rev* **1**, 215–222.

Wintergerst ES, Jelk J, Rahner C & Asmis R (2000) Apoptosis induced by oxidized low density lipoprotein in human monocyte-derived macrophages involves CD36 and activation of caspase-3. *Eur J Biochem* **267**, 6050–6059.

Wyllie AH, Morris RG, Smith AL & Dunlop D (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J Pathol* **142**, 67–77.

Xu JZ, Yeung SY, Chang Q, Huang Y & Chen ZY (2004) Comparison of antioxidant activity and bioavailability of tea epicatechins with their epimers. *Br J Nutr* **91**, 873–881.

Yang L & Sinensky MS (2000) 25-Hydroxycholesterol activates a cytochrome c release-mediated caspase cascade. *Biochem Biophys Res Commun* **278**, 557–563.

Zhu H, Takahashi Y, Xu W, Kawajiri H, Murakami T, Yamamoto M, Iseki S, Iwasaki T, Hattori H & Yoshimoto T (2003) Low density lipoprotein receptor-related protein-mediated membrane translocation of 12/15-lipoxygenase is required for oxidation of low density lipoprotein by macrophages. *J Biol Chem* **278**, 13350–13355.