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Attenuation of monocyte adhesion and oxidised LDL uptake in luteolin-treated human endothelial cells exposed to oxidised LDL

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Oxidative modification of LDL is causally involved in the development of atherosclerosis and occurs *in vivo* in the blood as well as within the vascular wall. The present study attempted to explore whether polyphenolic flavonoids influence monocyte-endothelium interaction and lectin-like oxidised LDL receptor 1 (LOX-1) expression involved in the early development of atherosclerosis. The flavones luteolin and apigenin inhibited THP-1 cell adhesion onto oxidised LDL-activated human umbilical vein endothelial cells (HUVEC), while the flavanols of (−)epigallocatechin gallate and (+)catechin, the flavonols of quercetin and rutin, and the flavanones of naringin, naringenin, hesperidin and hesperetin did not have such effects. Consistently, Western blot analysis revealed that the flavones at 25 μM dramatically and significantly abolished HUVEC expression of vascular cell adhesion molecule-1 and E-selectin evidently enhanced by oxidised LDL; these inhibitory effects were exerted by drastically down regulating mRNA levels of these cell adhesion molecules. In addition, quercetin and luteolin significantly attenuated expression of LOX-1 protein up regulated in oxidised LDL-activated HUVEC with a fall in transcriptional mRNA levels of LOX-1. In addition, quercetin and luteolin clearly blunted oxidised LDL uptake by HUVEC treated with oxidised LDL. The results demonstrate that the flavones luteolin and apigenin as well as quercetin were effective in the different initial steps of atherosclerosis process by inhibiting oxidised LDL-induced endothelial monocyte adhesion and/or oxidised LDL uptake. Therefore, certain flavonoids qualify as anti-atherogenic agents in LDL systems, which may have implications for strategies attenuating endothelial dysfunction-related atherosclerosis.

Lectin-like oxidised LDL receptor-1: Luteolin: Monocyte adhesion: Oxidized LDL: Vascular endothelial cell adhesion molecule-1

Inflammation has been linked to atherogenesis and plaque disruption and it is well established that atherosclerosis is a chronic inflammatory process (Ross, 1999). Atherosclerotic plaques consist of lipids, heavy infiltrates of inflammatory cells of macrophages and T-lymphocytes, smooth muscle cells and extracellular matrix (Tiong & Brieger, 2005). Endothelial dysfunction that initiates with the induction of cell adhesion molecules is one of the early events that lead to the development of the inflammatory reaction associated with atherosclerosis (Chia, 1998; Price & Loscalzo, 1999). Up regulation of adhesion molecules promotes the attachment of circulating mononuclear cells to the endothelium and facilitates their migration into the subendothelial space, which is an essential initial step in the formation of a perivascular inflammatory infiltrate (Price & Loscalzo, 1999; Lutters *et al.* 2004; Tiong & Brieger, 2005). Oxidized LDL that has been implicated in the atherosclerotic plaque rupture (Robbesyn *et al.* 2004), is known to play versatile pathological roles potentially involved in atherogenesis, including an induction of adhesion molecules in the endothelium (Chan, 1998). Pro-inflammatory cytokines, such as IL-1 and TNF-α released by incubating with oxidised LDL, up regulate the expression of cell adhesion molecules (Ohta *et al.* 2005).

Lectin-like oxidised LDL receptor 1 (LOX-1) is a major endothelial receptor for oxidised LDL and is deemed to play a pro-atherogenic role in atherosclerosis by internalizing and degrading oxidised LDL through a receptor-mediated pathway (Mehta & Li, 2002). Expression of LOX-1 is induced by many inflammatory cytokines as well as oxidised LDL (Cominacini *et al.* 2000; Hofnagel *et al.* 2004). Uptake of oxidised LDL through LOX-1 induces reactive oxygen species and activates NF-κB (Cominacini *et al.* 2000). In addition to oxidised LDL binding, LOX-1 has been shown to function as a cell adhesion molecule involved in inflammation (Honjo *et al.* 2003), suggesting the possible contribution of LOX-1 to the atherosclerotic pathogenesis.

Based on the literature evidence that polyphenolic flavonoids enhance LDL resistance to oxidation and attenuate

Abbreviations: DiI, 1.1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; LOX-1, lectin-like oxidised LDL-receptor-1; VCAM-1, vascular cell adhesion molecule-1.

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oxidised LDL-induced endothelial dysfunction (Jeong *et al.* 2005), the present study attempted to examine differential effects of diverse flavonoids including luteolin and quercetin on leukocyte-endothelial cell interactions and oxidised LDL uptake in oxidised LDL-exposed human umbilical vein endothelial cells (HUVEC). The participation of endothelial cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, and of LOX-1 in wielding these effects was elucidated.

Materials and methods

Materials

Fetal bovine serum (FBS), trypsin and penicillin-streptomycin were purchased from BioWhittker Co. (San Diego, CA, USA). Cell growth medium and supplements, flavonoids (flavanols, (−)epigallocatechin gallate and (+)catechin; flavonols, quercetin and rutin; flavanones, naringin, naringenin, hesperidin, hesperetin; flavones, luteolin and apigenin) and M199 chemicals were obtained from Sigma Chemical (St. Louis, MO, USA) as were all other reagents, unless specifically stated elsewhere. All flavonoids were solubilized by dimethyl sulfoxide for culturing with cells; the final culture concentration of dimethyl sulfoxide was $\leq 0{\cdot}5$ %, in which dose a lack of cytotoxic effect was observed. Endothelial viability was tested to rule out a non-specific cytotoxicity of flavonoids used in the present study (see Choi *et al.* 2003; Jeong *et al.* 2005).

Plasma LDL preparation and modification

Human plasma LDL was prepared by a discontinuous density gradient ultracentrifugation as previously described (Basu *et al.* 1976; Kang *et al.* 2002). The prepared plasma LDL obtained from human normo-lipidaemic 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 after isolation. Protein concentration of the plasma LDL fraction was determined by the Lowry method (Lowry *et al.* 1951) and concentrations of TAG and total cholesterol were measured by using diagnostic kits (Asan Pharmaceutical Co., Seoul, Korea). The contents of total protein, TAG, total cholesterol and phospholipid in the prepared LDL fraction were all in their appropriate ranges.

Fully oxidised LDL was prepared by incubating with 10 μM-$CuSO_4$ in F-10 medium at 37°C for 24 h. The extent of LDL oxidative modification was regularly determined using thiobarbituric acid reactive substances and electrophoretic mobility assay (Jeong *et al.* 2005). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)/labelled oxidised LDL was prepared by incorporating DiI (Molecular Probes Inc., Eugene, OR, USA) dissolved in dimethyl sulfoxide into oxidised LDL (Voyta *et al.* 1984).

Cell culture of human umbilical vein endothelial cells and THP-1

HUVEC were isolated using collagenase (Worthington Biochem. Co., 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 % FBS, 2 mM-glutamine, 100 U/ml penicillin, 0·1 mg/ml streptomycin and growth supplements (0·75 mg/ml human epidermal growth factor and 0·075 mg/ml hydrocortisone) at 37°C humidified atmosphere of 5 % CO_2 in air. Cells were passaged at confluence and used within ten passages. Endothelial cells were confirmed by their cobblestone morphology and uptake of modified LDL (Voyta *et al.* 1984).

Human monocytic cell line THP-1 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA, USA) and grown in HEPES-buffered RPMI-1640 containing 10 % FBS, 2 mM-glutamine, 100 U/ml penicillin and 0·1 mg/ml streptomycin. Cultures were maintained at 37°C in humidified atmosphere of 5 % CO_2 in air and cells were weekly passaged (1 : 4 ratio).

HUVEC cultured containing 25 mM-HEPES-buffered M199 with 10 % FBS were pre-treated with each tested flavonoid (25 μM) overnight prior to the exposure to 0·1 mg cholesterol/ml oxidised LDL. Subsequently, flavonoid- and oxidised LDL-treated HUVEC were incubated for the time period specifically stated in the experimental protocols.

Cell adhesion assay

HUVEC were cultured at a density of $7{\cdot}0 \times 10^4$ cells on a four-well glass chamber slide containing 25 mM-HEPES-buffered M199 with 10 % FBS. THP-1 cells were grown in RPMI-1640 medium containing 10 % FBS. HUVEC were pre-treated with each tested flavonoid (25 μM) overnight prior to the 5 h exposure to 0·1 mg cholesterol/ml oxidised LDL. THP-1 cells were labelled for 30 min with 5 μM-calcein-AM (Molecular Probes Inc.). All tested flavonoids did not show HUVEC toxicity significantly at concentrations ≤ 25 μM (Choi *et al.* 2003). Accordingly, all the flavonoids at non-toxic concentrations were used for culture experiments with oxidised LDL. The labelled THP-1 ($5{\cdot}0 \times 10^5$) were seeded onto confluent HUVEC treated with flavonoids and/or oxidised LDL and incubated for 1 h. Co-cultured cells were washed and the images were obtained at 485 nm excitation and 538 nm emission using a SPOT II digital camera-attached fluorescence microscope with Spot II data acquisition software (Diagnostic Instrument, Livingston, UK).

Western blot analysis

Whole cell extracts were obtained from HUVEC in a lysis buffer containing 10 mg/ml β-mercaptoethanol, 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 electrophoresed on 8 % or 12 % SDS-PAGE gels and transferred onto a nitrocellulose membrane. Non-specific binding was blocked by soaking the membrane in a buffer (0·5 M-Tris-HCl (pH 7·5), 1·5 M-NaCl and 0·1 % Tween 20) containing 5 % non-fat dry milk for 3 h. The membrane was incubated for 3 h with a primary antibody (rabbit polyclonal anti-human VCAM-1 (1 : 1000; Santa Cruz Biotech. Inc., Santa Cruz, CA, USA); goat polyclonal anti-human E-selectin (1 : 500; R & D System, Minneapolis, MN, USA); mouse anti-human LOX-1 (1 : 500; a gift from Dr. Sawamura, Japan); polyclonal rabbit anti-human LOX-1 (1 : 1000, Santa Cruz Biotech. Inc.)). After five washes with the buffer, the membrane was then incubated for 1 h with a goat anti-rabbit IgG or rabbit anti-goat IgG conjugated to horseradish peroxidase

(1 : 7500–10 000 dilution; Jackson Immuno Research Lab., West Grove, PA, USA). The protein levels of VCAM-1, E-selectin and LOX-1 were determined by using Supersignal West Pico chemiluminescence detection reagents (Pierce Biotech. 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 Biotech. Inc.) was also performed for the comparative control.

Immunocytochemistry

After endothelial cells were thoroughly washed with Tris buffered saline, cells were incubated for 20 min with 20 % normal goat serum in Tris buffered saline to block any non-specific binding. After washing fixed cells twice with Tris buffered saline, rabbit anti-human VCAM-1 (1 : 100 dilution), goat anti-human E-selectin (1 : 100 dilution) or rabbit anti-human LOX-1 (1 : 100 dilution) was added to cells and incubated overnight at 4°C. Cells were washed with Tris buffered saline and incubated with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1 : 10 000 dilution; Sigma Co.) or cyanin-conjugated donkey anti-goat IgG (1 : 10 000 dilution; Rockland, Gilbertville, PA, USA) as a secondary antibody. Images were obtained by an Olympus BX51 fluorescent microscope (Olympus Optical Co., Tokyo, Japan).

RT-PCR analysis

Total RNA was isolated from HUVEC using a commercially available Trizol reagent kit (Invitrogen Life Technol., Carlsbad, CA, USA) after culture protocols. The RNA (5 μg) was reversibly transcribed with 10 000 units of RT and 0·5 mg/ml oligo-$(dT)_{15}$ primer (Bioneer Co., Daejeon, Korea). The expressions of the mRNA transcripts of VCAM-1 (forward primer: 5′-ATGCCTGGGAAGATGGTCGTGA-3′, reverse primer: 5′-TGGAGCTGGTAGACCCTCGCTG-3′), E-selectin (forward primer: 5′-ATCATCCTGCAACTTCACC-3′, reverse primer: 5′-ACACCTCACCAAACCCTTC-3′), LOX-1 (forward primer: 5′-TTACTCTCCATGGTGGTGGTGCC-3′, reverse primer: 5′-AGCTTCTTCTGCTTGTTGCC-3′), and β-actin (forward primer: 5′-GACTACCTCATGAAGATC-3′, reverse primer: 5′-GATCCACATCTGCTGGAA-3′) were evaluated by RT-PCR as previously described (Park *et al.* 2003; Choi *et al.* 2004). The PCR was performed in 50 μl 10 mM-Tris-HCl (pH 8·3), 25 mM-$MgCl_2$, 10 mM-dNTP, 100 units *Taq* DNA polymerase, 0·1 μM each primer and was terminated by heating at 70°C for 15 min. After thermocycling and electrophoresis of the PCR products (5 μl) on 1 % agarose-formaldehyde gel containing ethidium bromide (0·5 μg/ml) for 1 h at 50 V, the bands were visualized using an UV transilluminator (Amersham Pharmacia Biotech., Piscataway, NJ, USA) and gel photographs were obtained using Polaroid Type 667 positive/ negative films. The absence of contaminants was routinely checked by the RT-PCR assay of negative control samples without a primer addition.

Endothelial uptake of oxidised LDL

DiI-labelled oxidised LDL was used to visualize the uptake of oxidised LDL by endothelial cells and macrophages (Jaakkola *et al.* 1988). After incubation of cells with each tested flavonoid, cells were treated with 0·03 mg protein/ml DiI-labelled oxidised LDL for 4 h in the absence and presence of 17-fold and 25-fold excess unlabelled oxidised LDL to determine specific uptake of oxidised LDL. After the end of incubation, the cells were rinsed with PBS containing 0·05 % Tween 20 and fixed in 4 % ice-cold formaldehyde for 30 min. Cellular distribution of DiI was analysed by an Olympus BX51 fluorescent microscope (Olympus Optical Co.) using a rhodamine filter set.

Data analysis

The results are presented as means with their standard errors for each treatment group in each experiment. Statistical analyses were conducted using Statistical Analysis Systems statistical software package version 6.12 (SAS Institute Inc., Cary, NC, USA). Significance was determined by one-way ANOVA followed by Duncan multiple range test for multiple comparisons. P values $<0·05$ were considered statistically significant.

Results

Adhesion of monocytes to oxidised LDL-activated endothelial cells

In vitro adhesion data obtained using the monocyte calcein-AM staining technique showed that there were few monocytes adhered to unactivated HUVEC free of oxidised LDL, whereas a marked staining on the HUVEC solely exposed to oxidised LDL for 5 h was observed (Fig. 1). This indicates a marked increase in THP-1 monocyte adherence to the oxidised LDL-activated HUVEC. However, the treatment of oxidised LDL-exposed cells with 25 μM-luteolin or apigenin significantly blocked the monocyte adherence. In contrast, the flavanols of (−)epigallocatechin gallate and (+)catechin, flavonols of quercetin and rutin, and flavanones of naringin, naringenin, hesperidin and hesperetin, at 25 μM did not inhibit the adhesion of THP-1 to oxidised LDL-activated endothelial cells.

Expression of cell adhesion molecules in oxidised LDL-treated endothelial cells

The observed blockade of mononuclear leukocyte recruitment onto the oxidised LDL-exposed vascular endothelium by the flavones, luteolin and apigenin, is deemed to be mediated via an inhibition of cell adhesion molecule induction. Western blot analysis revealed that treatment with 0·1 mg/ml oxidised LDL caused VCAM-1 induction in HUVEC with a maximum expression at 5 h after incubation (Fig. 2(A)) and this effect was inhibited by 25 μM-luteolin and apigenin (Fig. 2(B)). As expected, the exposure to other flavonoid subtypes, flavanols, flavonols and flavanones did not significantly suppress VCAM-1 expression induced by oxidised LDL ($P>0·05$, Fig. 2(B)).

Immunocytochemical assay using a VCAM-1 antibody was used to confirm differential effects of diverse flavonoids on the oxidised LDL-induced VCAM-1 expression. There was significant staining in HUVEC exposed to oxidised LDL (Fig. 3(A)). Pre-addition of luteolin to oxidised LDL-exposed

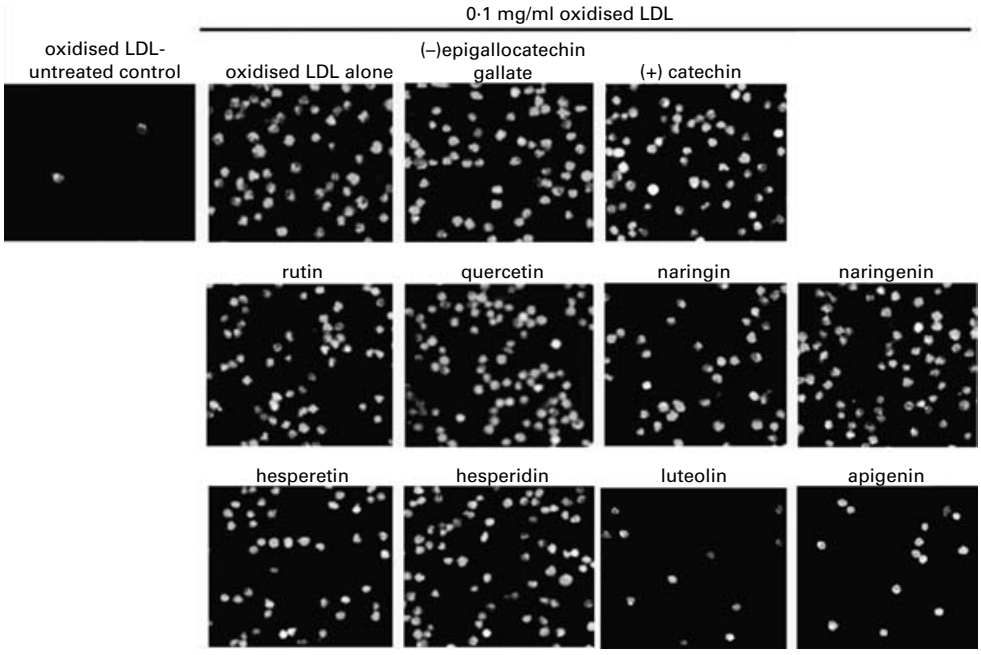

Fig. 1. Inhibition by luteolin and apigenin of calcein AM-labelled THP-1 monocyte adhesion to oxidized LDL-treated human umbilical vein endothelial cells (HUVEC). HUVEC were pre-treated with each test flavonoid (25 μM) and activated by 0·1 mg/ml oxidized LDL for 5 h and were co-cultured with THP-1 monocytes for 1 h. Microphotographs (five independent experiments) were obtained using a fluorescence microscopy. Magnification 200-fold.

cells suppressed the staining for VCAM-1. In contrast, the staining data revealed that all of (−)epigallocatechin gallate, rutin, quercetin and hesperetin did not mitigate the expression of VCAM-1 induced by oxidised LDL for 5 h (Fig. 3(A)). When the flavone apigenin was added in concentrations between 1 and 25 μM, this VCAM-1 induction was attenuated with pharmacologically inhibitory doses being ≥10 μM ($P<0·05$; Fig. 3(B)). In addition, ≥25 μM-luteolin significantly inhibited oxidised LDL-induced VCAM-1 expression.

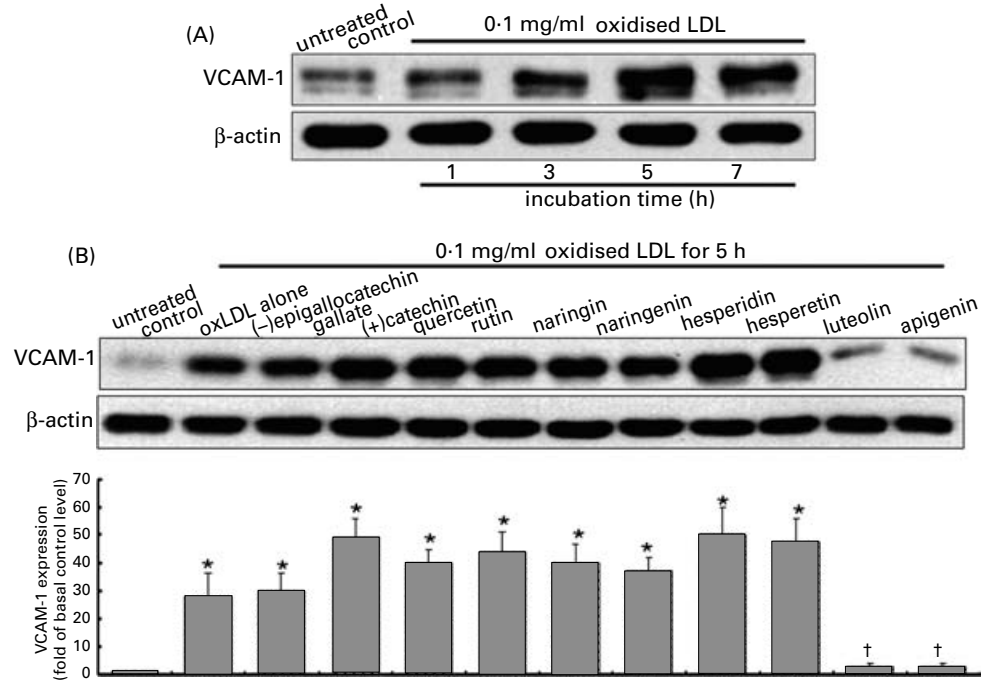

Fig. 2. Time course of vascular cell adhesion molecule-1 (VCAM-1) protein expression (A) and inhibitory effects of flavonoids on expression levels of VCAM-1 (B) in human umbilical vein endothelial cells (HUVEC) incubated with 0·1 mg/ml oxidised LDL (oxLDL). Total HUVEC protein extracts were electrophoresed on 8 % SDS-PAGE gels, followed by Western blot analysis with a primary antibody against VCAM-1. β-Actin protein was used as an internal control. Bands are representative of five independent experiments. The bar graphs (mean values with their standard errors; n 5) represent quantitative densitometric results of upper bands. *$P<0·05$, relative to untreated control incubation (value 1). †$P<0·05$, relative to oxidised LDL-alone incubation.

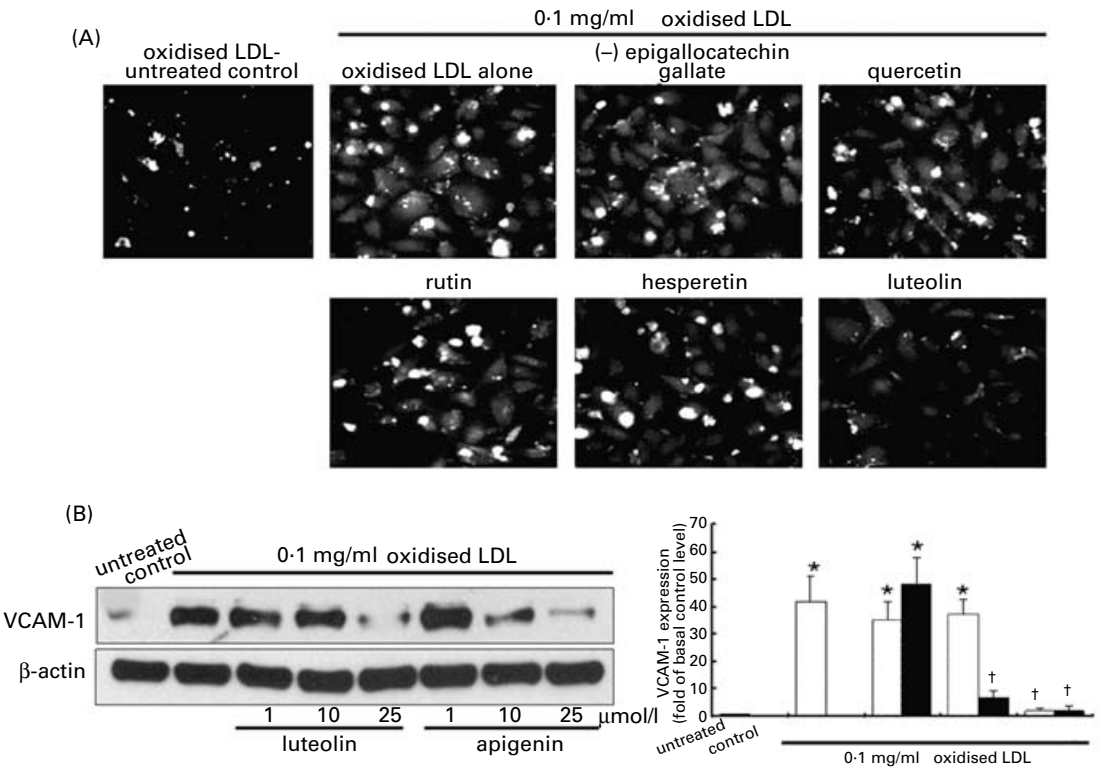

Fig. 3. Representative microphotographs showing effects on vascular cell adhesion molecule-1 (VCAM-1) expression of human umbilical vein endothelial cells (HUVEC) pre-treated with 25 μM-flavonoids and exposed to 0·1 mg/ml oxidised LDL for 5 h (A), and Western blot data showing dose–response of luteolin (□) and apigenin (■) to VCAM-1 expression (B). After fixation, the antibody localization (A) was detected by immunocytochemical staining with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. These microphotographs are representative of four independent slides. Magnification 200-fold. For Western blot analysis (B), total HUVEC protein extracts were electrophoresed on 8 % SDS-PAGE gels, followed by Western blot analysis with a primary antibody against VCAM-1. β-Actin protein was used as an internal control. Bands are obtained from four independent experiments. The bar graphs (mean values with their standard errors; *n* 4) represent quantitative densitometric results of left bands. **P*< 0·05, relative to untreated control incubation (value 1). †*P*< 0·05, relative to oxidised LDL-alone incubation.

Expression of another cell adhesion molecule, E-selectin, was also elucidated by Western blot analysis and immunocytochemical staining technique. Expression of E-selectin protein was noticeably enhanced in oxidised LDL-activated cells over the quiescent cells (Fig. 4(A)). Oxidized LDL-exposed cells treated with a pharmacological dose of 25 μM-luteolin proved complete inhibition of expression of E-selectin, whereas the other tested flavonoids did not visibly inhibit its expression. The immunocytochemical staining results supported the Western blot data (Fig. 4(B)), showing full inhibition of E-selectin induction in 25 μM-luteolin- and oxidised LDL-treated HUVEC.

Oxidized LDL-stimulated transcription of cell adhesion molecules

There were significantly weak signals for the basal mRNA expression of VCAM-1 and E-selectin in quiescent cells (Fig. 5). The RT-PCR data showed that oxidised LDL drastically enhanced mRNA expression of these adhesion molecules at 4 to 5 h after incubation (Fig. 5(A)). However, the mRNA levels of VCAM-1 and E-selectin in luteolin-treated cells were significantly and fully dropped off (Fig. 5(B)). Indeed, this was consistent with a marked attenuation of expression of these adhesion molecule proteins by flavones shown in Figs. 2–4. These results imply that flavone-type flavonoids inhibit the expression of cell adhesion molecule proteins via a direct modulation at their gene transcriptional levels.

Inhibition of lectin-like oxidised LDL receptor 1 induction by quercetin and luteolin

In oxidised LDL-exposed endothelial cells, a heavy staining was observed with a LOX-1 antibody, indicative of elevated expression of LOX-1, a major endothelial receptor for oxidised LDL (Fig. 6). When HUVEC were pre-treated with 25 μM-luteolin, the induction of LOX-1 was markedly attenuated. In addition, the induction was substantially diminished by the flavonol quercetin. In contrast, the flavanol (−)epigallocatechin gallate and the flavanone hesperidin and its aglycone hesperetin had no such effects (Fig. 6). Western blot data revealed similar results to those obtained from the experimental protocols using immunocytochemical staining techniques, revealing that oxidised LDL highly induced LOX-1 at 4 h after treatment (Fig. 7(A)). Accordingly, flavonol-type quercetin and flavone-type luteolin were deemed to be antagonists to this induction of LOX-1 by oxidised LDL ($P < 0{\cdot}05$; Fig. 7(B)).

We tested whether the attenuation of expression of LOX-1 protein by quercetin and luteolin was exerted at its gene transcriptional level. The basal mRNA expression of LOX-1 protein was very low in untreated cells (Fig. 7(C)). Oxidized LDL

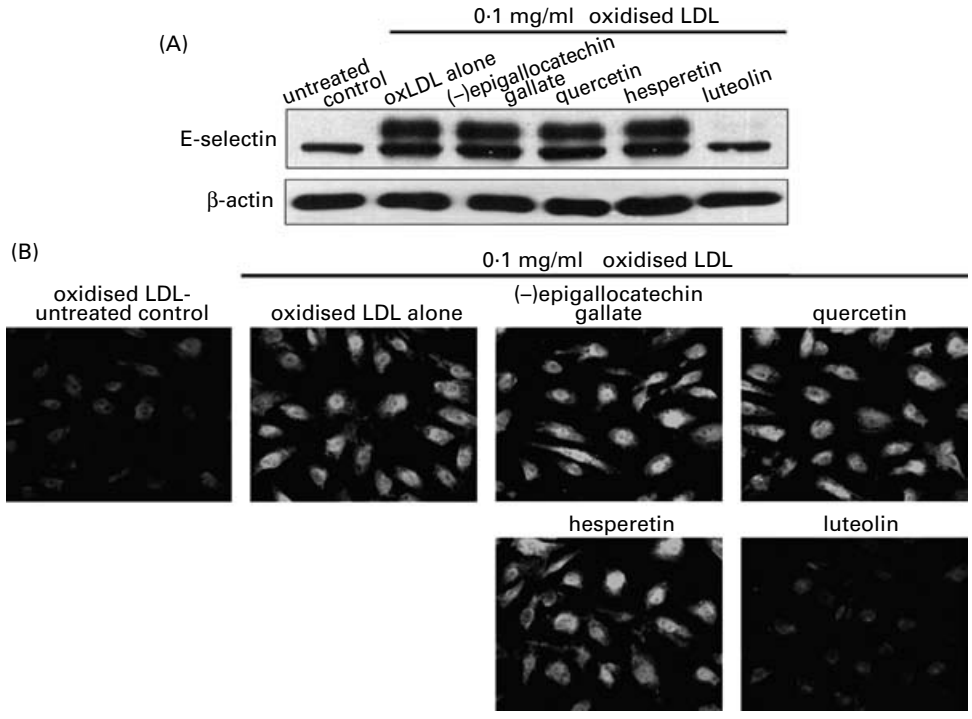

Fig. 4. Western blot analysis (A) and immunostaining (B) showing inhibitory effects of luteolin and apigenin on the protein expression level of E-selectin in human umbilical vein endothelial cells (HUVEC) incubated with 0·1 mg/ml oxidised LDL (oxLDL) for 5 h. Total HUVEC protein extracts were electrophoresed on 12 % SDS-PAGE gels, followed by Western blot analysis with a primary antibody against E-selectin or β-actin as an internal control. Bands are obtained from four independent experiments. Immunocytochemical staining was performed with cyanin-conjugated donkey anti-goat IgG. These microphotographs are representative of four independent slides. Magnification 200-fold.

induced expression of LOX-1 mRNA ($P<0·05$), while this expression in quercetin- or flavone-treated cells was significantly and fully diminished. Indeed, this was consistent with a marked attenuation of expression of LOX-1 protein by quercetin and luteolin, as shown in Figs. 6 and 7(B).

Uptake of oxidised LDL in endothelial cells

When HUVEC were pre-treated with each tested flavonoid (25 μM) and incubated with 0·03 mg/ml DiI-labelled oxidised LDL for 4 h, there was striking cytoplasmic staining in oxidised LDL-exposed cells, indicating marked DiI distribution in the cytoplasm due to uptake of oxidised LDL (Fig. 8). This receptor-mediated uptake of oxidised LDL was highly diminished by incubation with excess unlabelled oxidised LDL, suggesting that the uptake of oxidised LDL was specifically mediated through an up-regulation of oxidised LDL receptors. Luteolin, unlike (−)epigallocatechin gallate, hesperidin and hesperetin, effectively inhibited endothelial uptake of fluorescent DiI-labelled oxidised LDL, implying that this flavonoid may blunt the endothelial oxidised LDL receptors. Consistently, luteolin noticeably blocked the up-regulation of LOX-1 expression induced by oxidised LDL (Figs. 6 and 7(B)). In addition, with quercetin, the uptake of oxidised LDL was fairly suppressed possibly via a blockade of LOX-1 protein on the membrane surface of endothelial cells.

Discussion

Numerous studies have previously shown that polyphenolic compounds have considerable antioxidant abilities under various oxidative circumstances (Choi *et al.* 2005; Rackova *et al.* 2005; Teixeira *et al.* 2005). There is compelling evidence that the distinct structures of these compounds can partially explain their antioxidant activities (Rackova *et al.* 2005; Teixeira *et al.* 2005). Polyphenolic flavonoids are shown to delay LDL oxidation mainly through their antioxidant capacity (Jeong *et al.* 2005; Ruel *et al.* 2005). This implies that consumption of flavonoids may be effective in preventing or delaying the progression of atherosclerosis (Ruel *et al.* 2005). Dietary wine phenolics efficiently protect hypercholesterolaemic hamsters against aortic fatty streak accumulation (Auger *et al.* 2005). In addition, polyphenolic flavonoids suppress atherosclerotic development in apo E-deficient mice and in hypercholesterolaemic rabbits (Fuhrman *et al.* 2005). These findings propose that flavonoids can confer protection against the atherogenicity induced by vascular injury of oxidants, such as oxidised LDL.

It is believed that flavonoids may hamper early events in the atherosclerotic process. However, definite mechanisms underlying this protection against early atherogenic process are not fully understood. Modulation of monocyte adhesion onto endothelium could be one of the mechanisms by which flavonoids exert anti-atherogenicity in the early stages of atherosclerosis. In this report, the flavone-type flavonoids at 25 μM significantly inhibited THP-1 monocyte adhesion to oxidised LDL-activated endothelial cells, at least in part via an expression inhibition of cell adhesion molecules of VCAM-1 and E-selectin. It was shown that apigenin at ≥ 10 μM was highly potent in significantly blocking VCAM-1 expression. Flavanols, flavonols and flavanones did not have such

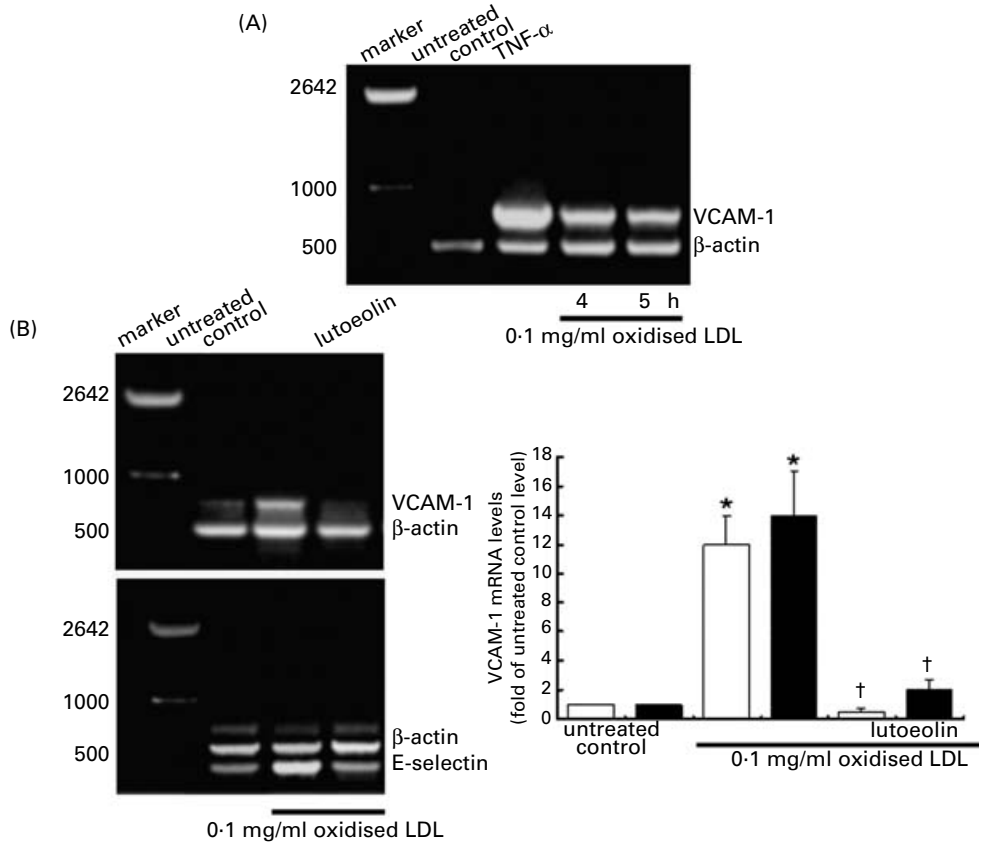

Fig. 5. RT-PCR data showing the expression time course of vascular cell adhesion molecule-1 (VCAM-1) mRNA (A) and the steady state mRNA transcriptional levels of VCAM-1 (□) and E-selectin (■) with oxidised LDL (B). Confluent human umbilical vein endothelial cells (HUVEC) were incubated with 25 μM-luteolin for 1 h and exposed to 0·1 mg/ml oxidised LDL for 4 h. The transcriptional level of VCAM-1 mRNA of HUVEC incubated with TNF-α for 6 h was used for comparison (A). β-Actin was used as an internal control for the co-amplification with VCAM-1 and E-selectin. The respective bands are extracted from four separate experiments. The bar graphs (mean values with their standard errors; *n* 4) represent quantitative results of respective panel B bands. **P*<0·05, relative to untreated control incubation (value 1). †*P*<0·05, relative to oxidised LDL-alone incubation.

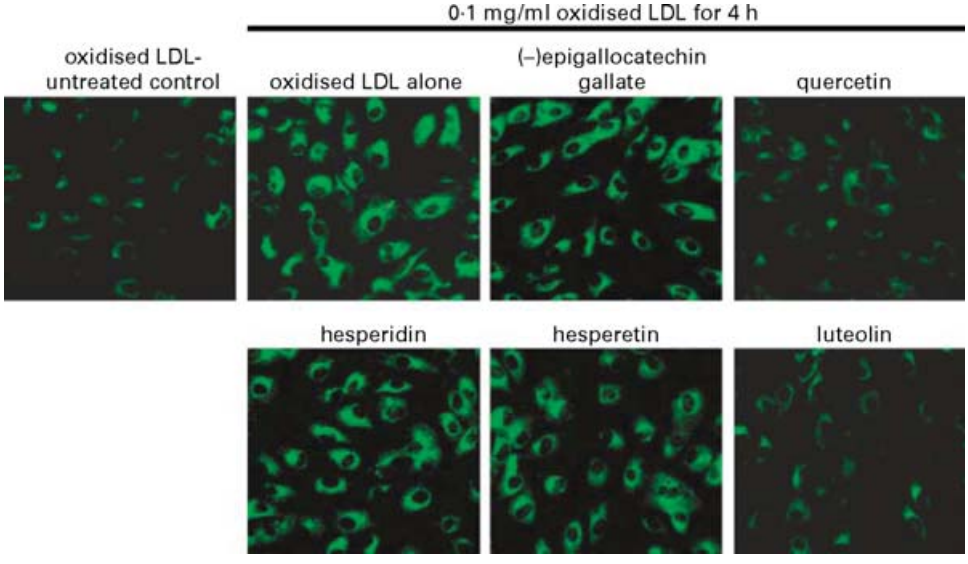

Fig. 6. Microphotographs for effects on lectin-like oxidised LDL receptor-1 expression of human umbilical vein endothelial cells pre-treated with 25 μM-flavonoids and exposed to 0·1 mg/ml oxidised LDL for 4 h. The antibody localization was detected by immunocytochemical staining with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. These microphotographs are obtained from five individual slides. Magnification 200-fold.

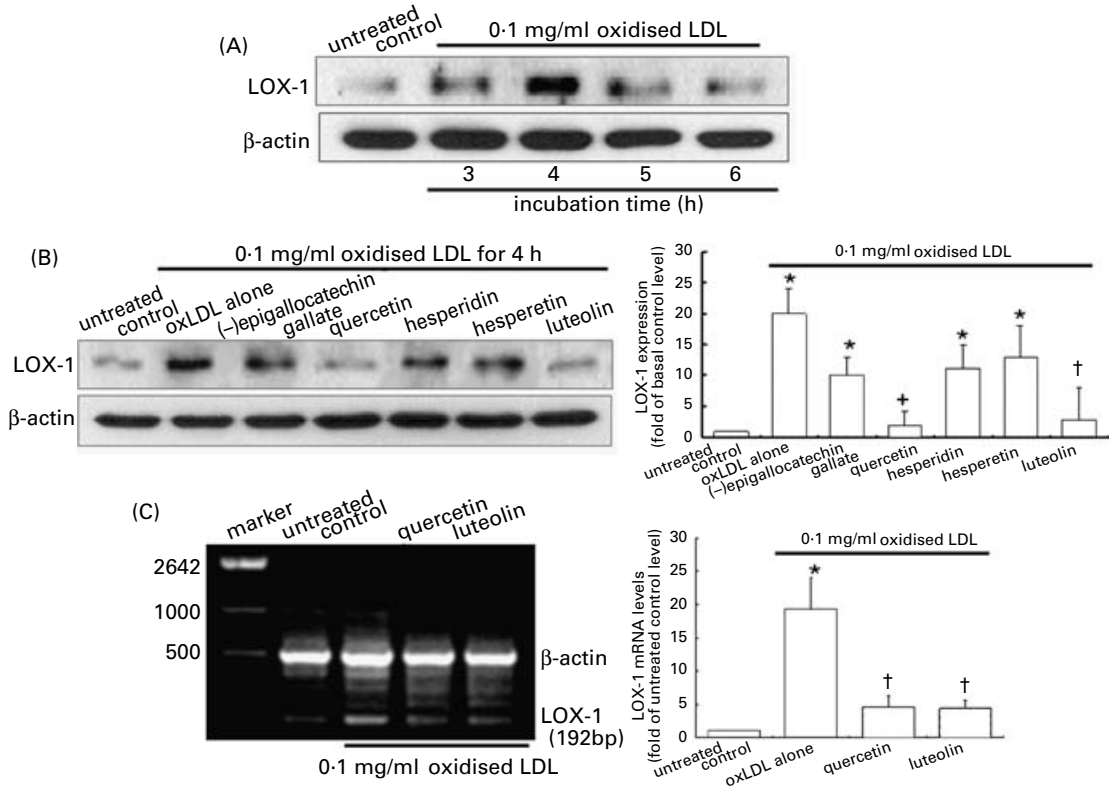

Fig. 7. Time course of lectin-like oxidised LDL receptor-1 (LOX-1) protein expression with 0·1 mg/ml oxidised LDL (A) and inhibitory effects of quercetin and luteolin on LOX-1 protein expression (B) and transcriptional levels of LOX-1 mRNA (C). Confluent human umbilical vein endothelial cells (HUVEC) were incubated with each tested flavonoid (25 μM) for 1 h and exposed to 0·1 mg/ml oxidised LDL for 4 h. For Western blot analysis, total HUVEC protein extracts were electrophoresed on 8 % SDS-PAGE gels, followed by Western blot analysis with a primary antibody against LOX-1. Bands are representative of five independent experiments. For RT-PCR, β-actin was used as an internal control for the co-amplification with LOX-1. The gel bands are representative of four separate experiments. The bar graphs (mean values with their standard errors; *n* 4) represent quantitative results of respective gel bands in panels B and C. **P*<0·05, relative to untreated control incubation (value 1). †*P*<0·05, relative to oxidised LDL-alone incubation.

inhibitory activity and the rutinose moiety present in flavanone glycosides, i.e. naringin and hesperidin, did not facilitate the blockade of monocyte adhesion on the activated endothelium.

The ability of flavones luteolin and apigenin to prevent oxidised LDL-induced expression of cell adhesion molecule could be attributed to their antioxidant capacity. It has been demonstrated that oxidative stress up regulates cell adhesion molecule expression that is inhibited by antioxidant systems (Kokura *et al.* 2001; Deem & Cook-Mills, 2004; Llurba *et al.* 2004; Segui *et al.* 2005). Classical antioxidants such as vitamin

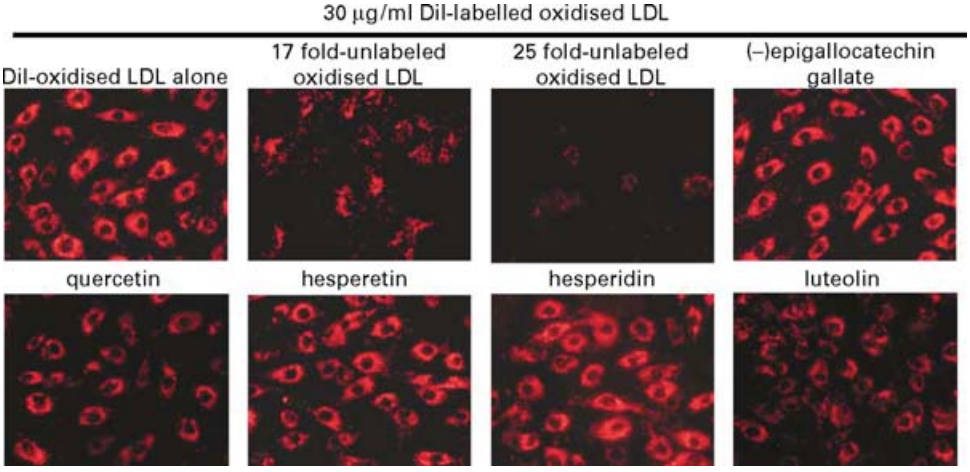

Fig. 8. Inhibition of oxidised LDL uptake by flavonoid-treated human umbilical vein endothelial cells exposed to 1.1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labelled oxidised LDL for 4 h. Cells were incubated with 0·03 mg/ml DiI-labelled oxidised LDL in the absence and presence of 0·5–0·75 mg/ml unlabelled oxidised LDL to determine specific uptake of oxidised LDL. Cellular distribution of DiI was analysed by a fluorescent microscope equipped with a rhodamine filter set. These microphotographs are representative of five individual slides. Magnification 200-fold.

E have been shown to inhibit endothelial interactions with leukocytes induced by oxidised LDL (Yoshida *et al.* 2000; Meydani, 2004). In the present study flavanols and flavonols did not influence the oxidised LDL-induced monocyte adhesion and cell adhesion molecule expression. Our previous study demonstrated that the flavanol (−)epigallocatechin gallate and the flavonol quercetin were powerful in scavenging a stable free radical 1, 1-diphenyl-Z-picrylhydrazyl (DPPH), whereas the flavones luteolin and apigenin had a weak radical scavenging activity (Choi *et al.* 2003). Thus, it is unlikely in this study that the antioxidant activity of flavonoids contributes to their blockade of endothelial adhesion molecule induction by oxidised LDL.

It is not yet determined how flavones down regulated endothelial expression of VCAM-1 and E-selectin in response to oxidised LDL. However, it is clearly found that the blockade of endothelial expression induction of VCAM-1 and E-selectin by luetolin and apigenin was mediated through mechanism(s) responsive to the flavones. The inhibitory mechanism(s) of these flavones were assumed from the notion that they may interrupt signalling cascades, leading to transcriptional activation of cell adhesion molecules in the presence of oxidised LDL. Flavones significantly attenuated transcriptional mRNA expression of VCAM-1 and E-selectin. A NF-κB-dependent mechanism is deemed to be one of the mechanisms by which flavones play an important role in inhibiting oxidised LDL-induced expression of leukocyte adhesion molecules. It has been documented that oxidised LDL exerts a biphasic effect on the redox-sensitive NF-κB, which can be activated, thereby up regulating pro-inflammatory gene expression, such as adhesion molecules, tissue factors and scavenger receptor LOX-1 (Robbesyn *et al.* 2004). Our previous study has shown that quercetin and flavones attenuated or blocked nuclear translocation of p65 and the DNA-binding activity of NF-κB stimulated by pro-inflammatory cytokine TNF-α, which in turn attenuated cell adhesion molecule expression at the transcriptional levels (Robbesyn *et al.* 2004). Luteolin abolished lipopolysaccharide-induced increase in phosphorylation of the NF-κB inhibitory protein IκB-α and pro-inflammatory cytokine production in murine macrophages (Xagorari *et al.* 2001). However, the mechanisms by which flavones block endothelial expression of VCAM-1 and E-selectin are still uncertain in this report.

The present study revealed that luteolin and quercetin significantly mitigated transcriptional mRNA expression of inducible LOX-1 and hence endothelial uptake of oxidised LDL. This study did not elucidate detailed mechanisms involved in inhibiting LOX-1 induction. Nevertheless, it is speculated that oxidised LDL appears to enhance endothelial LOX-1 expression through oxidative stress-sensitive pathways via NF-κB (Robbesyn *et al.* 2004). Oxidized LDL may up regulate the nuclear activity of NF-κB through signalling pathways of mitogen-activated protein kinases (Mehta *et al.* 2004; Nishimura *et al.* 2004). Since LOX-1 inhibition is associated with an attenuation of atherosclerosis and ischaemic injury (Li *et al.* 2002), LOX-1 may be a novel target for drug therapy. Aspirin inhibits oxidised LDL-mediated LOX-1 expression and interferes with the effects of oxidised LDL in intracellular signalling through p38 mitogen-activated protein kinase activation (Mehta *et al.* 2004). Oxidized LDL binding to LOX-1 in articular chondrocytes activated NF-κB at least in part through a production of intracellular reactive oxygen species, which was reduced by the antioxidant L-ascorbic acid (Nishimura *et al.* 2004). Collectively, it can be speculated that the access of quercetin and luteolin as antioxidant agents to putative binding proteins may modulate oxidised LDL-mediated activation of NF-κB signalling cascades and LOX-1 expression through interrupting activation of mitogen-activated protein kinase. It should be noted that the flavanol (−)epigallocatechin gallate and the flavanone hesperetin did not mitigate LOX-1 expression and the subsequent uptake of oxidised LDL, despite their antioxidant activity. Thus, oxidised LDL-mediated LOX-1 expression appears to be exerted through unknown mechanism(s) exclusively responsive to quercetin and luteolin, independent of oxidative stress-sensitive and NF-κB-dependent mechanisms.

There is a close link between LOX-1 and cell adhesion molecule expression, which may play an important role in atherosclerosis and pathological endothelial dysfunction (Kobayashi *et al.* 2005). Incubation of endothelial cells with high glucose increased human monocyte adhesion to endothelium through a LOX-1-dependent signalling mechanism, suggesting a role for LOX-1 as a mediator of the stimulatory effect of high glucose on monocyte adhesion (Li *et al.* 2003). In this study oxidised LDL induced LOX-1 expression both at the gene and protein levels. The binding of oxidised LDL to the up-regulated LOX-1 is deemed to elicit the induction of cell adhesion molecules and, hence, endothelial dysfunction with uptake of oxidised LDL. Accordingly, oxidised LDL-related atherosclerotic effects of the flavone luteolin may be partly caused by inhibition of LOX-1-mediated cell adhesion molecules, possibly via LOX-1-dependent signalling mechanisms. However, the possibility cannot be ruled out that oxidised LDL acts as a direct mediator inducing cell adhesion molecules and stimulating subsequent monocyte adhesion. Quercetin did not inhibit the oxidised LDL-induced VCAM-1 and E-selectin both at the gene and protein levels. This implies that quercetin did not influence the direct induction of cell adhesion molecules by oxidised LDL regardless of inhibition of LOX-1 expression.

In summary, the present study has revealed that the capability of flavonoids to prevent early processes such as endothelial expression of inducible adhesion molecules and LOX-1 and endothelial uptake of oxidised LDL, which is known to be involved in the development of atherosclerosis, differs among flavonoid subtypes. The flavones luteolin and apigenin blocked *in vitro* monocyte adhesion on the oxidised LDL-activated endothelium and the activation of expression of cell adhesion molecules and LOX-1. Quercetin attenuated the oxidised LDL uptake via an inhibition of endothelial LOX-1 expression. The expression of these proteins was selectively modulated at transcriptional levels by quercetin and flavones but not by flavanols and flavanones in response to oxidised LDL. This observation provides indications for potential anti-atherogenic action of flavones and quercetin on the oxidised LDL-mediated process associated with atherosclerosis.

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