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Natural phenylpropanoids inhibit lipoprotein-induced endothelin-1 secretion by endothelial cells

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Abstract

There is increasing evidence that oxidized low-density lipoproteins (Ox-LDL) might be involved in the pathogenesis of atherosclerosis and it has been reported that polyphenols inhibit LDL peroxidation and atherosclerosis. Endothelin-1 (ET-1) is a potent vasoconstrictor peptide isolated from endothelial cells and it induces smooth muscle cell proliferation. ET-1 secretion is increased in atheroma and induces deleterious effects such as vasospasm and atherosclerosis. The goal of this study was to test the effect of four natural phenolic compounds against copper-oxidized LDL (Cu-LDL)-induced ET-1 liberation by bovine aortic endothelial cells (BAEC). The tested compounds were phenylpropanoid glycosides previously isolated from the aerial parts of *Marrubium vulgare* L. (acteoside 1, forsythoside B 2, arenarioside 3 and ballotetroside 4). ET-1 secretion increased when cells were incubated with Cu-LDL but the compounds **1**–**4** inhibited this increase. These results were confirmed by quantitative-polymerase chain reaction (QPCR) analysis. Since ET-1 plays an important role in atherosclerosis development, our work suggests that the tested phenylpropanoids could have a beneficial effect in inhibiting atherosclerosis development.

Introduction

Oxidatively-modified low-density lipoproteins (LDLs) have an important role in the initiation and progression of atherosclerosis (Steinberg 1997). Modification of LDLs through oxidative damage may increase their atherogenicity by increasing their receptor-mediated uptake by cells in the intima of blood vessels, leading to creation of foam cells in the subendothelium, an early feature of atherosclerotic plaque (Duriez et al 1994).

Hayek et al (1997) reported that consumption of polyphenols (quercetin or catechin) reduced the progression of atherosclerosis in apolipoprotein E-deficient mice. The inhibition of atherosclerosis development was associated with reduced susceptibility of LDLs to ex-vivo oxidation. Furthermore, the consumption of red wine polyphenols in man also reduces the susceptibility of LDLs to oxidation in-vivo (Nigdikar et al 1998). The ability of polyphenols to inhibit the oxidation of LDLs demonstrates their potential as chain-breaking antioxidants (Brown et al 1998) and might involve a role in saving α-tocopherol (Jessup et al 1990), the main endogenous antioxidant of LDLs.

Endothelin-1 (ET-1) is a 21-amino-acid peptide that acts as a potent vasoconstrictor (Yanagisawa et al 1988) by activating specific receptors expressed in vascular smooth muscle cells (VSMC) (Simonson 1993). Recent findings suggest that ET-1 relates hypercholesterolaemia to the development of atherosclerosis (Fan et al 2000). ET-1 induces phenotypic changes that lead to the development of the plaque, including migration and proliferation of VSMC (Janakidevi et al 1992) and induction of adhesion molecules' expression by endothelial cells (McCarron et al 1993). Furthermore, inhibition of ET-1 receptors in animal models of atherosclerosis reduces the formation of fatty streak (Kowala et al 1995; Barton et al 1998). In addition, in man and animals, the increase in circulating ET-1 is related to the development of atherosclerosis lesions (Miyauchi et al 1992; Haak et al 1994).

An intervention study in man (Cuevas et al 2000) showed a marked decrease of endothelial function with a high-fat diet. Endothelial function was restored with a

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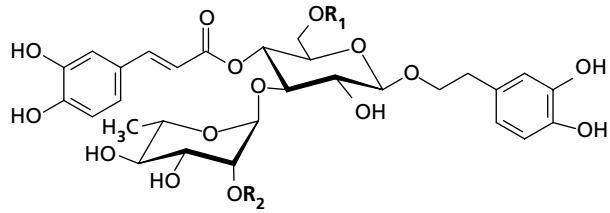
Mediterranean-type diet, containing fruits and vegetables, and further increased by supplementation with moderate wine intake.

Phenylpropanoid glycosides are phenolic compounds widely distributed in different plant families. We have isolated four phenylpropanoids from the aerial parts of *Marrubium vulgare* L. (Sahpaz et al 2002). These are glycoside derivatives (acteoside = verbascoside **1**, forsythoside B **2**, arenarioside **3** and ballotetroside **4**) (Figure 1), which are present in significant amounts, ca 5% of dry weight of the plant.

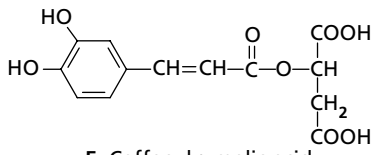
Our recent studies (Martin-Nizard et al 2003) showed that the phenylpropanoid glycosides **1**–**4**, containing two catechols, are strong inhibitors of both copper (Cu^{2+})- and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced LDL oxidation in-vitro. Therefore, these molecules inhibit LDL oxidation by scavenging free radicals independent of any capacity to act as Cu^{2+} chelators.

Oxidized LDLs (Ox-LDLs) have numerous biological effects, such as cytotoxicity, that might accelerate atherosclerosis (Esterbauer et al 1993). Furthermore, we demonstrated that compounds **1**–**4** preserved the morphological aspect of bovine aortic endothelial cells (BAEC) during their incubation with air-oxidized LDL (air-Ox-LDL). They reduced the accumulation of aldehydes in the cultured medium during the incubation of BAEC with air-Ox-LDL and prevented cellular lactate dehydrogenase (LDH) leakage during this period. These data suggest that natural phenylpropanoids inhibit air-Ox-LDL-induced cellular toxicity and that inhibition of lipid peroxidation could be a key mechanism in the cytoprotective effect of these molecules (Martin-Nizard et al 2003).

Thus, the aim of this work was to test the effect of compounds **1**–**4** against copper-oxidized LDL (Cu-LDL)-induced BAEC ET-1 liberation.



	R_1	R_2
1 Acteoside	H	H
2 Forsythoside B	β-D-apiose	H
3 Arenarioside	β-D-xylose	H
4 Ballotetroside	β-D-apiose	α-L-arabinose



5 Caffeoyl-L-malic acid

Figure 1 Chemical structures of studied compounds.

Materials and Methods

Isolation of phenylpropanoids

Phenylpropanoids **1**–**4** were isolated from *Marrubium vulgare* aerial parts as previously described (Sahpaz et al 2002). The structures of all compounds were established on the basis of their physical ($[\alpha]_D$) and spectral data (MS, ^1H and ^{13}C NMR) and confirmed in comparison with those of authentic samples previously isolated in the laboratory of Pharmacognosy (University of Lille 2).

LDL preparation

LDLs (density range 1.019–1.063) were apyrogenically prepared from freshly drawn blood from normolipidaemic male subjects by sequential ultracentrifugation as described previously (Havel et al 1955) and then extensively dialysed against phosphate-buffered saline (PBS) containing 0.01% ethylenediaminetetraacetic acid (EDTA) at 4°C. The LDLs used for oxidative modification by Cu^{2+} were dialysed for 24 h against PBS at 4°C. Protein content was determined by Peterson's method (Peterson 1977). Cu-LDLs were prepared by incubating 1 mg mL^{-1} LDL for 24 h with 1.66 μmol L^{-1} $CuSO_4$ at 37°C. Oxidation was stopped by adding 20 μmol L^{-1} EDTA. Typical thiobarbituric acid-reactive substances (TBARS) values (Ohkawa et al 1979) were around 0.1 nmol (mg protein)$^{-1}$ for non-oxidized LDL (LDL) and 46–47 nmol (mg protein)$^{-1}$ for Cu-LDL.

Cell culture

Bovine aortic endothelial cells (BAEC) were isolated as previously described (Gospodarowicz et al 1976) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, 600 ng mL^{-1} glutamine and 100 U mL^{-1} penicillin. BAEC were used at passages 4–8.

ET-1 secretion

BAEC were subcultured in 6-well plates. Sub-confluent cells were treated for 24 h with either 10 μmol L^{-1} compounds **1**–**4**, vehicle (0.01% v/v methanol) or PBS, following a change of media containing the same concentration of compounds **1**–**4** in the presence or absence of 100 μg mL^{-1} of LDL or Cu-LDL in lipoprotein-deficient serum for 16 h. At the end of the treatment period, medium was collected and ET-1 concentration was determined using a radioimmunoassay (RIA) kit (Amersham). Results were normalized to cellular protein content in all experiments.

RNA extraction and analysis

Total cellular RNA was extracted from cells using Trizol (Life Technologies, France). For quantitative-polymerase chain reaction (QPCR), total RNA was reverse transcribed using random hexameric primers and Superscript

reverse transcriptase (Life Technologies, France). cDNAs were quantified by real-time PCR on an MX 4000 (Stratagene), using specific primers for bovine ET-1: 5′ T-GCT-GCT-CTT-CCC-TGA-TGG-3′ and 5′-GGC-ATC-TCT-TCC-TGT-GGA-CTG-TCG-3′, bovine 36B4: 5′-CAT-GCT-GAA-CAT-CTC-CCC-CTT-CTC-C 3′ and GGG-AAG-GTG-TAA-TCA-GTC-TCC-ACA-G 3′. PCR amplification was performed in a volume of 25 μL containing 100 nmol L^{-1} of each primer, 4 mmol L^{-1} $MgCl_2$, the Brilliant Quantitative PCR Core Reagent Kit mix as recommended by the manufacturer (Stratagene) and SYBR Green 0.33X (Sigma-Aldrich). The conditions were 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. E-1 mRNA levels were subsequently normalized to 36B4 mRNA.

Statistics

Results were expressed as mean ± s.d. when a minimal number of three independent experiments were performed in triplicate. The comparison of data between more than two groups was analysed by analysis of variance comparison. Significant differences were then subjected to post-hoc analysis by using the Sheffe test. A value of $P < 0.05$ was accepted as statistically significant.

Results and Discussion

Lipoproteins increase ET-1 secretion

LDL and Cu-LDL significantly increased ET-1 secretion in comparison with the control (PBS 100%): 110.3 ± 3.7% with LDL ($P < 0.01$) and 127.2 ± 3.2% with Cu-LDL ($P < 0.001$), respectively (Figure 2A).

Effect of lipoproteins and compounds 1–4 on ET-1 secretion

Compounds **1**–**4** did not significantly modify ET-1 secretion in comparison with the control (methanol 0.01% v/v) (Figure 2B), while LDL and Cu-LDL increased this secretion (Figure 2A).

Compounds **1**–**4** were capable of completely abolishing the capacity of Cu-LDL to induce ET-1 secretion (Figure 3). Cu-LDL (100 μg) increased basal ET-1 secretion (127.2 ± 3.2% versus control 100 ± 2.7%) whereas compounds **1**–**4** with 100 μg Cu-LDL significantly decreased the secretion induced by Cu-LDL (98.2 ± 4.5, 100.5 ± 4.5, 107.1 ± 3.8 and 102.3 ± 3.5 (all $P < 0.001$), respectively).

Quantitative real time PCR analysis showed an ET-1 mRNA induction when Cu-LDL was incubated with BAEC (2.1 ± 0.25 versus control 1 ± 0.1) (Figure 4). This induction reached the basal state when BAEC were incubated with Cu-LDL and compounds **1**–**4** (1.1 ± 0.15, 0.9 ± 0.3, 1.2 ± 0.25 and 1.1 ± 0.2 (all $P < 0.05$), respectively).

Ox-LDLs have numerous biological effects, such as cytotoxicity, which might accelerate atherosclerosis (Esterbauer et al 1993). We have shown (Furman et al 1999) that air-Ox-LDLs or Cu-LDLs have differential toxicities towards endothelial cells: air-Ox-LDLs are highly toxic inducing an increase in LDH release and decrease in cellular ATP and reduced glutathione (GSH) concentrations. As we had previously shown that

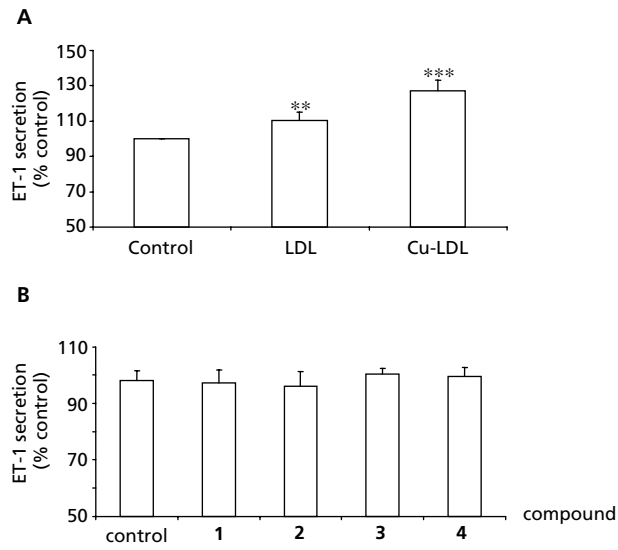


Figure 2 A. Effect of non-oxidized low-density lipoprotein (LDL) and copper-oxidized LDL (Cu-LDL) on ET-1 secretion. Bovine aortic endothelial cells (BAEC) were incubated for 16 h with LDLs (100 μg mL^{-1}) or control (PBS) in medium containing 10% v/v lipoprotein-deficient serum. Results are presented as a percentage of ET-1 secretion measured in control situation (medium with PBS 100 μL). ET-1 concentration was measured in the culture media by RIA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, absence of LDL vs presence of LDL, n = 3. Three independent experiments were performed in triplicate. B. Effect of compounds **1**–**4** on ET-1 secretion. BAEC were incubated for 40 h with 10 μmol L^{-1} of compounds **1**–**4** or vehicle (methanol 0.01% v/v). Results are presented as a percentage of ET-1 secretion measured in control situation (medium with 0.01% methanol). Three independent experiments were performed in triplicate.

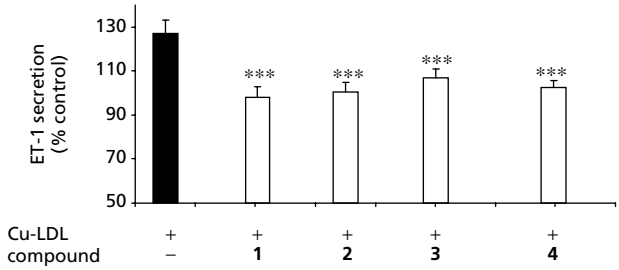


Figure 3 Compounds **1**–**4** inhibit lipoprotein-induced ET-1 secretion. Bovine aortic endothelial cells (BAEC) were incubated for 24 h with 10 μmol L^{-1} of compounds **1**–**4** or vehicle (methanol 0.01% v/v) followed by a change to medium containing the same treatment as before plus Cu-LDL 100 μg mL^{-1} with 10% lipoprotein-deficient serum for 16 h. ET-1 concentration was measured in the culture media by RIA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Cu-LDL plus compounds **1**–**4** vs Cu-LDL without compounds **1**–**4**, n = 3. Three independent experiments were performed in triplicate.

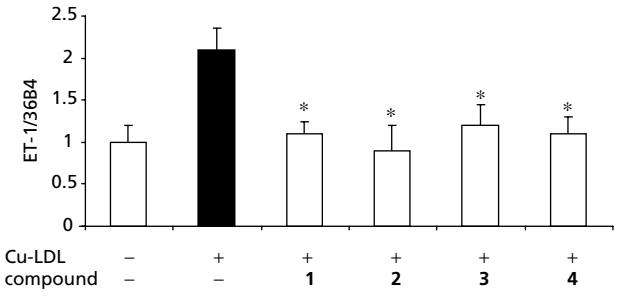


Figure 4 ET-1 gene expression is induced by Cu-LDL but the induction was abolished by compounds **1**–**4**. Quantitative PCR analysis of ET-1 was performed on RNA isolated from bovine aortic endothelial cells (BAEC) treated or not for 24 h with 10 μmol L^{-1} of compounds **1**–**4**, vehicle (0.01% methanol) followed by a change to medium containing the same treatment as before plus Cu-LDL 100 μg mL^{-1} or PBS in 10% lipoprotein-deficient serum for 16 h. ET-1 mRNA levels were normalized to bovine 36B4 mRNA and are expressed relative to the levels in untreated cells. Results are means ± s.d. of triplicate determinations, representative of three independent experiments. *$P < 0.05$, absence vs presence of compounds **1**–**4** (*t*-test).

Cu-LDL induced ET-1 secretion in endothelial cells without cytotoxicity (Sqalli-Houssaini et al 1994, Martin-Nizard et al 2002), we used Cu-LDL in this study. To demonstrate whether or not the effect of tested molecules on ET-1 synthesis was due to non-specific cytotoxicity, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide) or LDH assay was performed on the BAEC after each experiment.

Firstly, we verified the capacity of LDLs to stimulate ET-1 liberation. LDL and Cu-LDL increase ET-1 concentration in the culture medium (Figure 2A), confirming previous data (Sqalli-Houssaini et al 1994, Martin-Nizard et al 2002). The secretion induced by LDL was lower than that induced by Cu-LDL but was, however, significant. These data confirm the results indicating that a long period of incubation with BAEC induces a modest LDL oxidation (Morgan et al 1993). The stimulation of LDL receptors by LDL could also induce ET secretion (Sqalli-Houssaini et al 1994).

Then we showed that compounds **1**–**4** did not stimulate ET-1 liberation (Figure 2B). Treatment with the tested molecules (**1**–**4**) and Cu-LDL did not significantly modify cellular LDH release and did not induce any morphological cellular alteration. Furthermore, there was no degradation in the mitochondria function (data not shown). Taken together, these results suggest that compounds **1**–**4** and LDLs were not cytotoxic in BAEC.

We have previously shown that phenylpropanoids **1**–**4** inhibit LDL oxidation in-vitro (Martin-Nizard et al 2003). In this work we showed that these compounds were capable of completely abolishing the capacity of Cu-LDL to induce ET-1 secretion (Figure 3). Corder et al (2001) found that polyphenols from red wine made from Cabernet Sauvignon grapes decreased ET-1 synthesis in cultured BAEC by suppressing transcription of the ET-1 gene.

To confirm these results, Q-PCR analysis was performed on BAEC, which showed that ET-1 expression was increased by Cu-LDL (Figure 4). This induction was abolished by compounds **1**–**4**.

Conclusion

It has been demonstrated that LDL oxidation in the subendothelial compartment is a primary event in atherogenesis. Oxidized LDLs increase ET-1 secretion in endothelial cells.

Since ET-1 plays an important role in atherosclerosis development, our work suggests that the phenylpropanoids isolated from the aerial parts of *Marrubium vulgare* L. could have a beneficial effect by inhibiting atherosclerosis development.

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