Inhibition of Human Low Density Lipoprotein and High Density Lipoprotein Oxidation by Oligostilbenes from Rhubarb

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The objective of the present study was to elucidate the beneficial properties of ampelopsin B (1) and ϵ -Viniferin (2), two oligostilbenes isolated from rhubarb, toward cardiovascular disease by protecting human lipoproteins against lipid peroxidation. In low density lipoprotein (LDL) oxidation, both 1 and 2 exert an inhibitory activity against Cu^{2+} -, 2,2'-azobis-(2-amidinopropane) hydrochloride (AAPH)-induced, as exhibited by prolongation of lag time from 52 to 118 and 136 min, respectively, and also increasing the lag time 38 to 105 and 128 min in high density lipoprotein (HDL) oxidation for 1 and 2, respectively, at the concentration of 3.0 μM . In generation of thiobarbituric acid reactive substances (TBARS), compounds 1 and 2 inhibited LDL oxidation mediated by either catalytic Cu^{2+} or thermo-labile radical initiator (AAPH) in a dose-dependent manner with IC_{50} values of 3.6 and 6.0 μM for 1, and 1.7 and 3.2 μM for 2, respectively. In addition, compounds 1–2 also showed strong ability to protect HDL oxidation induced by both Cu^{2+} and AAPH with low IC_{50} values. The results suggest that oligostilbenes 1–2 may have a role in preventing lipoprotein oxidation.

Key words rhubarb; ampelopsin B; ϵ -Viniferin; lipoprotein

Lipoproteins and lipoprotein oxidation play an important role in development, progression, and pathobiochemistry in many age-related disorders, such as atherosclerosis, cardiovascular disease, coronary heart disease, and aging in general, by free radical attack and reactive oxygen species to lipids, proteins, and DNA.^{1–3} Numerous prospective cohort investigations have demonstrated the high density lipoprotein (HDL) plasma levels are inversely correlated with coronary artery disease and the risk of atherosclerosis.^{4,5} The protective effects of HDL include reverse cholesterol transport, anti-inflammatory and antioxidative actions by protecting low density lipoprotein (LDL) from oxidation *via* its main Apo A-I and antioxidant enzymes.^{6–8} Oxidatively modified LDL within the arterial wall or in serum is believed to be readily taken up by macrophages leading to the formation of lipid laden foam cells, with possible build-up of atherosclerotic plaque,⁹ and may cause thrombosis.¹⁰ Atherosclerosis arterial wall contain increased levels of copper and iron ions which contribute to the oxidation of lipoproteins by the generation of free radicals.¹¹ A beneficial effect of antioxidants on reactive oxygen species generation *in vitro* has been clearly demonstrated,¹² although *in vivo* studies still lack conclusive results.⁷

As a part of our screening program to find antioxidant compounds from natural sources, we have attempted to determine the human lipoprotein oxidation inhibitory constituents of Korean medicinal plants. Rhubarb is the rhizomes of *Rheum undulatum* L., *R. palmatum*., *R. tanguticum* MAXIM., *R. officinale* BAILL., and *R. coreanum* NAKAI, an important and well-known medicinal origin plant which has been used in traditional medicine for the treatment of blood stagnation and as well as a purgative agent.¹³ Previously, a number of natural stilbene and anthraquinone derivatives were identified as the main components with many biological activities such as anti-inflammatory, anti-diabetic, anti-allergic, cytotoxicity, anti-carcinogenic and antioxidant.^{14–17} Among them, am-

pelopsin B (1) and ϵ -Viniferin (2) are the oligostilbenes with dimer-stilbene skeleton, which was shown to have inhibitory activity against cyclooxygenase, lipoxygenase, and tyrosinase.^{18,19} However, the protective effect on human lipoproteins against lipid peroxidations has not been studied. The aim of this investigation was to examine whether oligostilbenes (1, 2) inhibit LDL and HDL oxidation *in vitro* over a wide physiological and pharmacological range. LDL and HDL oxidation was induced by copper ion (Cu^{2+}) and azoinitiator (AAPH), and the subsequent increase in conjugated diene formation and in lipid peroxidation products was monitored.

MATERIALS AND METHODS

Plant Material The cultivated Korean rhubarb rhizome was purchased in July 2006 from the Yuseong herbal drug market, Daejeon, Korea, and identified by Professor KiHwan Bae. A voucher specimen (CNU-1345) has been deposited in the herbarium of the College of Pharmacy, Chungnam National University, Korea.

Extraction and Isolation The dried and milled rhizomes of rhubarb (4.7 kg) were extracted with 20 l ethanol, three times. The ethanol extract was combined and concentrated to yield a residue (650 g), which was suspended in water and then successively partitioned with hexane, ethyl acetate (EtOAc) and butanol (BuOH) to afford 32.7 g hexane-, 308 g EtOAc-, and 108 g BuOH-soluble fractions, respectively.

The EtOAc-soluble fraction was diluted with acetone, and then filter through filter paper to give precipitated powder (E1, 20.7 g) and acetone fraction (E2, 226.1 g). The E2 (100 g) was subjected to silica gel column chromatography, and eluted with CHCl_3 -MeOH (80:1 to 0:1) to separate into six fractions (E2.1–E2.6). The E2.6 was chromatographed on silica gel column with CHCl_3 -MeOH (5:1 to

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0:1) to divide into five fractions (E2.6.1—E2.6.5). Fraction E2.6.3 was further subjected to MPLC [ODS, 250×10 mm i.d., MeOH—H₂O (35:75)] to furnish compounds **1** (76 mg) and **2** (37 mg).

Ampelopsin B (1): Pale brown powder, mp: 205—207 °C; $[\alpha]_D^{25} +170.4^\circ$ ($c=0.10$, MeOH); UV $\lambda_{\max}$ (MeOH): 224 and 281 nm; IR $\nu_{\max}$ (KBr) cm^{-1} : 3340, 1590, 1510, 1150, 970; ¹H-NMR (400 MHz, acetone-*d*₆) δ : 7.09 (2H, d, $J=8.7$ Hz, H-2, 6), 6.93 (2H, d, $J=8.4$ Hz, H-2', 6'), 6.76 (2H, d, $J=8.4$ Hz, H-3', 5'), 6.63 (2H, d, $J=8.7$ Hz, H-3, 5), 6.42 (1H, d, $J=2.1$ Hz, H-12), 6.32 (1H, d, $J=1.8$ Hz, H-14'), 6.22 (1H, d, $J=2.1$ Hz, H-10), 6.05 (1H, d, $J=2.1$ Hz, H-12'), 5.72 (1H, d, $J=11.4$ Hz, H-7), 5.21 (1H, t, $J=3.9$ Hz, H-7'), 4.17 (1H, d, $J=11.4$ Hz, H-8), 3.57 (1H, dd, $J=3.9$, 17.5 Hz, H-8'), 3.20 (1H, br d, $J=17.4$ Hz, H-8'). ¹³C-NMR (100 MHz, acetone-*d*₆) δ : 160.5 (C-13'), 158.8 (C-4), 158.6 (C-13), 157.2 (C-11'), 156.7 (C-13), 156.1 (C-11), 142.7 (C-9'), 138.2 (C-1'), 134.8 (C-9), 131.1 (C-1), 130.1 (C-2', 6'), 128.6 (C-2, 6), 122.9 (C-14), 119.1 (C-10'), 116.1 (C-3, 5), 115.6 (C-3', 5'), 109.1 (C-14'), 105.5 (C-10), 101.6 (C-12), 95.8 (C-12'), 88.4 (C-7), 49.4 (C-8), 35.9 (C-7'), 33.9 (C-8').

ϵ -Viniferin (2): Pale brown powder, mp: 149—151 °C; $[\alpha]_D^{25} -47.0^\circ$ ($c=0.5$, MeOH); UV $\lambda_{\max}$ (MeOH): 220, 304 and 319 nm; IR $\nu_{\max}$ (KBr) cm^{-1} : 3340, 1610, 1570, 1510, 1330, 1260, 1160; ¹H-NMR (400 MHz, acetone-*d*₆) δ : 7.21 (2H, d, $J=8.4$ Hz, H-2', 6'), 7.17 (2H, d, $J=8.4$ Hz, H-2, 6), 6.91 (1H, d, $J=16.2$ Hz, H-7'), 6.83 (2H, d, $J=8.7$ Hz, H-3', 5'), 6.74 (2H, d, $J=8.7$ Hz, H-3, 5), 6.74 (1H, overlap, H-14'), 6.71 (1H, d, $J=16.2$ Hz, H-8'), 6.33 (1H, d, $J=2.1$ Hz, H-12'), 6.25 (3H, br s, H-10, 12, 14). ¹³C-NMR (100 MHz, acetone-*d*₆) δ : 162.5 (C-4'), 159.9 (C-11, 13), 159.6 (C-11'), 157.7 (C-4, 13'), 147.5 (C-9), 136.5 (C-9'), 133.9 (C-1), 130.2 (C-7'), 129.9 (C-1'), 128.7 (C-2', 6'), 128.0 (C-2, 6), 123.6 (C-8'), 119.9 (C-10'), 116.4 (C-3, 5), 116.2 (C-3', 5'), 107.1 (C-10, 14), 104.3 (C-14'), 102.2 (C-12), 96.9 (C-12'), 93.9 (C-7), 57.2 (C-8).

LDL and HDL Preparation Blood from healthy normolipemic donors was obtained by venipuncture and collected in EDTA-containing vacutainer tubes. LDL was prepared from plasma by sequential flotation ultracentrifugation as described previously.²⁰⁾ To isolate HDL, plasma was prepared by centrifugation at 3000 rpm for 10 min and thereafter used for the preparation of plasma lipoproteins. HDL was isolated from plasma by ultracentrifugation for 1.5 h with a vertical rotor.²¹⁾ After dialysis at 4 °C for 24 h against 10 mM phosphate-buffered saline (PBS) pH 7.4, HDL protein concentration (mg protein/ml) was determined as described by Lowry *et al.*²²⁾

Cu²⁺ Mediated HDL, LDL Oxidation The oxidation of lipoproteins was assessed by the formation of conjugated dienes determined as the change in UV absorbance at 232 nm. Briefly, HDL (150 $\mu\text{g/ml}$) or LDL (150 $\mu\text{g/ml}$) in PBS (pH 7.4) was pre-incubated with either the absence (control) or presence of tested compounds, and then Cu²⁺ (5 μM) was added to initiate the oxidation at 37 °C. Absorbance at 232 nm was continuously monitored at 10 min intervals for 5 h at 37 °C using a spectrophotometer (Shimadzu UV-1240, Tokyo, Japan). The lag time was measured as the intercept between the baseline and the tangent of the absorbance curve during the propagation phase.²³⁾

The oxidation of LDL or HDL to malondialdehyde (MDA)

was measured using the thiobarbituric acid reactive substances (TBARS) assay. Briefly, lipoproteins (100 $\mu\text{g/ml}$) in PBS (pH 7.4) were pre-incubated with various concentrations of tested compounds, and then Cu²⁺ was added to initiate the oxidation. The reaction mixture was incubated at 37 °C for 3 h and the reaction was terminated by adding 20% trichloroacetic acid (TCA) and 1% thiobarbituric acid (TBA). After boiling at 95 °C for 15 min, the mixture was centrifuged at 5000 rpm for 10 min. The absorbance of supernatant was measured at 532 nm.

Peroxyl Radicals Mediated Human Lipoproteins Oxidation The oxidation of lipoprotein by peroxyl radicals was performed by incubation of the HDL or LDL (150 $\mu\text{g/ml}$) at 37 °C in PBS (pH 7.4) with one of the thermo-labile radical initiators (AAPH) for 3 h. AAPH (10 μM) served as a source of hydrophilic peroxyl radicals. At the end of the incubation, samples were also assessed by measuring accumulation of lipid aldehydes (TBARS) as described above.

Statistical Analysis The results are expressed as mean values S.D. Statistical analysis was performed using oneway ANOVA. A $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Repeated column chromatography of the EtOAc-soluble fraction of the EtOH extract of rhubarb led to the isolation of two oligostilbenes **1** and **2** (Fig. 1). Their structures of isolated compounds were identified as ampelopsin B (**1**)²⁴⁾ and ϵ -Viniferin (**2**)^{25,26)} by comparison of physical chemistry data previously reported.

In this study, we attempted to examine their protective LDL and HDL against oxidation induced by Cu²⁺ and AAPH. The oxidation of human lipoproteins was assessed by the formation of MDA using TBARS assay. As shown in Table 1, both oligostilbenes **1** and **2** were strongly inhibitory against lipid peroxidation by not only metal ion Cu²⁺-induced but also peroxyl radical AAPH-induced in HDL with IC₅₀ values of 3.9 and 8.2 μM for **1**, and 2.4 and 5.7 μM for **2**, respectively. A comparison with positive controls showed Vitamin E inhibitive effect with IC₅₀ values exhibiting the activity of 4.3 and 10.2 μM , under Cu²⁺ and AAPH mediated oxidations, respectively. Interestingly, it was demonstrated that compounds **1** and **2** also exhibited significantly inhibitory activity against the oxidation of LDL initiated by Cu²⁺ in a dose-dependent manner, with IC₅₀ values of 3.0 and 1.7 μM , respectively. Subsequently, under AAPH mediated oxidation, it also exhibited LDL-oxidation protective activities with IC₅₀ values of 6.1 and 3.2 μM , respectively. Oth-

Fig. 1. Chemical Structures of Isolated Compounds **1** and **2**

Table 1. Effect of Tested Compounds on LDL-, HDL-oxidation by TBARS Method

Compounds	LDL-oxidation			HDL-oxidation		
	Lag time (min)	TBARS, IC ₅₀ (μM) ^{a)}		Lag time (min)	TBARS, IC ₅₀ (μM) ^{a)}	
		Cu ²⁺ -mediated	AAPH-mediated		Cu ²⁺ -mediated	AAPH-mediated
DMSO	52			38		
Ampelopsine B (1)	118	3.0±1.2*	6.1±1.8*	105	3.9±1.2*	8.2±2.0*
ε-Viniferin (2)	136	1.7±1.0*	3.2±1.3*	128	2.4±1.1*	5.7±1.9*
Vitamin E ^{b)}	75	20.9±2.4	ND	83	4.3±1.2	10.2±1.9

a) The values present mean ± S.D. of triplicate experiments; b) compounds used as positive control; **p* < 0.05 vs. vitamin E. ND: not determined.

erwise, in the same conditional experiment, α -tocopherol only showed inhibitory activity in Cu²⁺-mediated LDL oxidation with IC₅₀ value of 20.9 μM, and vitamin E was found to have no or only weak activity in an AAPH mediated experiment (data not shown).

In further study, the effect of **1** and **2** on the change of lag time was investigated. The formation of conjugated dienes represents the early peroxidation during lipoprotein oxidation, and the period of lag time was interpreted as the oxidation resistant capacity of LDL and HDL. The results in Fig. 2A illustrate the LDL oxidation kinetics induced by 5 μM Cu²⁺ in the absence and the presence of 3 μM tested compounds. Both **1** and **2** caused a significant modification of the kinetics of LDL oxidation producing an increase of lag time to 118 and 136 min, respectively, *versus* 52 min in control (with 1% DMSO, v/v), and lag phase of vitamin E retarded to 75 min (Table 1). Additionally, at low concentration, oligostilbenes **1** and **2** also exhibited strong ability to protect HDL oxidation induced by Cu²⁺, and achieved a significant prolongation of the lag time to 105 and 128 min, respectively, compared with control which was 38 min (Fig. 2B). In this experiment, the lag phase of vitamin E increased to 83 min (Table 1).

Based on the oxidation theory for atherosclerosis, dietary antioxidants have attracted considerable attention as preventive and therapeutic agents. There is large body of observational studies (demological, case-control or prospective and retrospective cohort) on the dietary antioxidant intake link to prevent cardiovascular disease progression.²⁷⁾ Consumption of dietary rich phenols and polyphenols can reduce the risk of heart disease by slowing the progression of atherosclerosis principally by protecting HDL and LDL from oxidation.²⁸⁾ Oligostilbenes **1** and **2** are phenolic compounds which possess five free aromatic-OH groups. These compounds have previously been tested for their antioxidant activity such as free radical scavenging and also their inhibitory effect against lipoxygenase,^{14,18)} which is an enzyme indicated in the participation of the LDL lipid peroxidation process during the early stage of plaque development.²⁹⁾

This study reports evidence that all tested compounds have a protective capacity to different extents on Cu²⁺-induced oxidation of human lipoproteins. A significant increase in lag time was observed when LDL or HDL was supplemented even with a low concentration of samples (3 μM). The comparable results in LDL, HDL oxidation demonstrated that oligostilbenes **1** and **2** have greater capacity to increase the lag phase of the conjugated diene process than vitamin E (Figs. 2A, B). In principle, the antioxidant effect was also ev-

Fig. 2. Conjugated Diene Effect of Compounds **1** and **2** on the Cu²⁺-Mediated LDL (A) and HDL (B) Oxidation

LDL (150 μg/ml) or HDL (150 μg/ml) in PBS (pH 7.4) was incubated with 5 μM Cu²⁺ at 37 °C in the presence or absence of 3 μM tested compounds **1** and **2**, and Vitamin E. Conjugated diene was measured by determining the absorbance at 234 nm continuously at 8 min intervals for 6 h.

ident in TBARS formation reduced by various sample concentrations after 180 min incubation. The IC₅₀ values were established as shown in Table 1, oligostilbenes **1** and **2** were more effective antioxidant in the metal-dependent pro-oxidant system than in the peroxy radical system, could be understood *via* chain-breaking mechanism by donation of phenolic hydrogen to lipoprotein bearing metal ion chelating properties. The antioxidant activities come from chemical structures themselves. Compounds **1** and **2** are dimer derivatives of resveratrol bearing mechanistic importance of the 4'-OH group, the acidity of this group and subsequent transfer of protons or hydrogen atoms to reactive species appear to be crucial to its antioxidant mechanism. All five hydroxyl hydrogens must be disordered and simultaneously act as H-bond donors and H-bond acceptors to accommodate this hydrogen-bonding scheme.³⁰⁾ This antioxidant mechanism is used by one of the most potent phenolic antioxidants found in nature: a form of vitamin E which transfers its phenolic proton to a propagating lipid peroxy radical to terminate the

chain reaction.³¹⁾ Ampelopsin B (**1**) has lost its ethylene double bond, which decreases the rotational energy for the phenyl group containing the 4'-OH group. Such information can be important for resveratrol accommodation in enzyme pockets or interaction with lipoproteins,³¹⁾ and may explain the lower antioxidant efficiency of **1** than ϵ -Viniferin (**2**).

There is a supposition that rhubarb could be used as an alternative therapy for heart disease, and thus oligostilbenes **1** and **2** and some other stilbenes would be able to play an important role in reducing oxidative stress, preventing the development and progression of atherosclerotic disease. In conclusion, our studies add significant evidence for the *in vitro* ability of ampelopsin B (**1**) and ϵ -Viniferin (**2**) to inhibit both LDL and HDL oxidation, and thus offer an additional natural antioxidant resource to prevent cardiovascular disease development.

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