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Antrodia Camphorata in Submerged Culture Protects Low Density Lipoproteins Against Oxidative Modification

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Abstract: *Antrodia camphorata* is well known in Taiwan as a traditional Chinese medicine. In this study, we have investigated the antioxidant properties of a fermented culture broth of *Antrodia camphorata* (FCBA) and the aqueous extracts of mycelia from *Antrodia camphorata* (AEMA) on the oxidative modification of human low-density lipoproteins (LDL), as induced by either copper sulfate ($CuSO_4$) or 2,2′-azo- bis(2-amidinopropane) hydrochloride (AAPH). Under such oxidant stress, FCBA and AEMA appear to possess antioxidant properties with respect to oxidation of LDL in a time- and concentration-dependent manner, as assessed by inhibition of thiobarbituric acid-reactive substances (TBARS) formation, conjugated diene production, and cholesterol degradation of oxidized LDL. In addition, both FCBA and AEMA exhibited a remarkable ability to rescue the relative electrophoretic mobility and fragmentation of the Apo B moiety of the oxidized LDL. Furthermore, FCBA and AEMA effectively protected the endothelial cells from the damaging effects of the $CuSO_4$-oxidized LDL. Our findings suggest that the antioxidant properties of *Antrodia camphorata* may also provide effective protection from atherosclerosis.

Keywords: *Antrodia camphorata*; Antioxidant; LDL; Atherosclerosis.

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Introduction

A new basidiomycete, *Antrodia camphorata* (Wu and Chiang, 1995), which occurs in the Polyporaceae (Aphyllophorales) and causes brown heart rot in *Cinnamonum kanehirai* hay (Lauraceae) in Taiwan, has been identified as a new genus of the *Antrodia* species (Wu *et al*., 1997). *A. camphorata* is rare and expensive, since it grows only on the inner heart-wood wall of *Cinnamonum kanehirai* and cannot be cultivated. It has been utilized in traditional Chinese medicine for the treatment of drug intoxication, diarrhea, abdominal pain, hypertension, skin itches, and liver cancer (Tsai and Liaw, 1985). However, very few biological activity tests has been reported.

There is increasing evidence that oxidation of low-density lipoprotein (LDL) plays a major role in the development of atherosclerosis. Oxidized LDL, a major risk factor for atherosclerosis, is taken up by arterial wall macrophages at an enhanced rate, leading to cellular cholesterol accumulation and foam cell formation (Steinberg *et al*., 1989; Aviram, 1993; Witztum, 1994). This atherogenicity of oxidized LDL also involves cytotoxicity against arterial wall cells, and proinflammatory and thrombotic properties (Yla-Herttuala *et al*., 1989; Berliner *et al*., 1995; Aviram, 1996). Supplementation with antioxidants is known to increase LDL resistance to oxidation both *in vitro* and *in vivo* (Esterbauer *et al*., 1991; Jialal and Grundy, 1992). Furthermore, inhibition of LDL oxidation can retard the development of atherosclerosis in animal models (Carew *et al*., 1987; Williams *et al*., 1992; Kleinvld *et al*., 1994), and increased intake of the antioxidants is associated with decreased incidence of new (Rimm *et al*., 1993) or recurrent (Stephens *et al*., 1996) cardiovascular disease, as well as reduced angiographic progression (Hodis, 1995). Therefore, it seems reasonable to suggest that antioxidants in foods and/or drugs may play a role in the prevention of atherosclerosis and cardiovascular diseases.

For 2000 years, medicinal mushrooms have been used in China to improve health and achieve longevity. *A. camphorata* has recently become popular as a remedy in Taiwan, as well as a source of physiologically beneficial mushrooms. In our previous study, *A. camphorata* was used for the inhibition of AAPH-induced oxidative hemolysis and lipid/protein peroxidation of normal human erythrocytes (Hseu *et al*., 2002). Interestingly, *A. camphorata* exhibits significant cytotoxicity against leukemia HL-60 cells but not against cultured human endothelial cells, suggesting that *A. camphorata* may have protective antioxidant and anticancer properties in humans (Hseu *et al.*, 2004). Other studies indicate that the antioxidant activity of *A. camphorata* may partially be a result of reducing sugars, predominantly components of the mycelia polysaccharide, because the antioxidant activity is reportedly concomitant with the development of the reducing power (Huang, 2000; Tanaka *et al*., 1988). A previous study, based on evaluations using different antioxidant test systems, has demonstrated an association between the antioxidant activity of *A. camphorata* and its polysaccharide, polyphenol, and triterpenoid contents (Song and Yen, 2002). Scientific interest in these active compounds (polysaccharides, polyphenols, and triterpenoids isolated from mushrooms) has recently been aroused due to evidence indicating their antioxidant, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties (Huang and Ferraro, 1992; Rao and Gurfinkel, 2000; Liu *et al.*, 1997; Zhu *et al*., 1999; Wasser and Weis, 1999; Ma *et al*., 1991).

In this study, we investigated the antioxidant and free radical scavenging activity of *A. camphorata* in terms of the inhibition of oxidative modification of LDL, induced by incubation with either copper sulfate ($CuSO_4$) or 2,2′-azo-bis[2-amidinopropane] dihydrochloride (AAPH). It has been reported that, compared to other *A. camphorata* extracts, the fermented culture broth of *A. camphorata* (FCBA) and aqueous extracts of the mycelia of *A. camphorata* (AEMA) harvested from submerged cultures are the most potent inhibitors of lipid peroxidation, possessing marked free radical scavenging activity (Song and Yen, 2002). Given the interesting biological activities and potential clinical applications, FCBA and AEMA were used for the study of the antioxidant properties.

Materials and Methods

Chemicals

Collagenase and BSA were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) (Wako Chemicals, Richmond, VA, USA), Trolox (Aldrich Chemical Co., Milwaukee, WI, USA), fetal bovine serum (FBS), M-199 medium and penicillin-streptomycin-neomycin (PSN) (GIBCO Laboratories, Grand Island, NY, USA) were sourced. Other materials were of reagent grade quality, and supplied either by Merck (Darmstadt, Germany) or Sigma Chemical Co.

Preparation of Fermented Culture Broth of A. camphorata (*FCBA*)

Culture of *A. camphorata* was inoculated on potato dextrose agar and incubated at 30ºC for 15–20 days. The whole colony was then excised and placed into the flask with 50 ml sterile water. After homogenization, the fragmented mycelia suspension was used as an inoculum. The seed culture was prepared in a 20 L fermentor (BioTop) agitated at 150 rpm, with an aeration rate of 0.2 vvm at 30ºC. Fifteen liters of a five-day-old culture of the mycelia inoculum was inoculated into a 250 L agitated fermentor. The conditions were the same as for the seed fermentation, with the exception of aeration rate (0.075 vvm). The fermentation product was then harvested at hour 331 and poured through non-woven fabric on a 20-mesh sieve to separate the deep-red fermented culture broth and the mycelia, before being centrifuged at 3000*g* for 10 minutes, followed by passage through a 0.2 μm filter. The stock solution was stored at −20ºC before analysis of its antioxidant properties. The culture broth was concentrated in a vacuum and freeze dried to form a powder. Dry matter yield of the culture broth was 9.8 g/L.

Preparation of Aqueous Extracts of Mycelia from A. camphorata (*AEMA*)

Mycelia in water were filtered three times through Whatman #1 paper before being air dried. For the preparation of aqueous extracts, all air-dried mycelia samples were ground and then shaken with 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl

(PBS) at a ratio of 1:25 (w/v) at 25ºC for 10 hours, and then centrifuged at 3000*g* for 10 minutes, followed by passage through a 0.2 μm filter (Hseu *et al.*, 2002). The aqueous extracts were concentrated in a vacuum and freeze dried to form a powder, which was stored at −20ºC before analysis of its antioxidant properties. The yield of mycelia was 1.1%.

LDL Isolation and Oxidation

Fresh human blood plasma was collected from fasting normal volunteers in tubes containing 0.01% EDTA. The plasma LDL was isolated by ultra-centrifugation at densities of 1.019–1.063 g/ml (Chang and Huang, 1990). The LDL fractions were pooled and dialyzed three times against fresh 10 mM PBS solution (pH 7.4) in the dark at 4ºC for 24 hours. All samples were subsequently stored in the dark at 4ºC in a nitrogen atmosphere. Oxidative modification of LDL was induced by incubation of LDL with either $CuSO_4$ or AAPH in the dark at 37ºC. FCBA or AEMA was added 30 minutes prior to the incubation mixture to test its capacity to block LDL oxidation under these conditions. After the incubation, conjugated diene and TBARS formation, cholesterol degradation, electrophoretic mobility, and Apo B fragmentation were measured as described below. The oxidation reaction was stopped by maintaining at 4ºC. Trolox was used as a positive control for the antioxidant activity tests.

Assay for Formation of Thiobarbituric Acid Reactive Substances (*TBARS*)

TBARS assay was used to estimate the generation of malondialdehyde (MDA) equivalents during LDL oxidation (Steinbrecher *et al.*, 1984). Oxidative modification of LDL was induced by incubation of LDL (50 μg/ml) at 37ºC with either 10 μM $CuSO_4$ or 1 mM AAPH for 6, 12 and 24 hours, respectively. The FCBA or AEMA was added to the incubation mixture to test the capacity to block LDL oxidation under these conditions. After oxidation, LDL was mixed with 1.5 ml of 0.67% TBA and 1.5 ml of 20% trichloroacetic acid. After heating at 100ºC for 30 minutes, the reaction was maintained at 25ºC for 30 minutes, and centrifuged for 15 minutes at 4ºC. The reaction product was assayed fluorometrically using an optical spectrofluorometer (F-2000, Hitachi), with excitation at 515 nm and emission at 553 nm. Freshly diluted tetraethoxypropane, which yields MDA, was used as a standard. Results are expressed as nmol MDA/mg protein.

Assay for Production of Conjugated Diene

Conjugated diene formation was determined as described by Esterbauer *et al*. (1989). LDL (50 μg protein/ml) was incubated with either 10 μM $CuSO_4$ or 1 mM AAPH in the presence or absence of FCBA/AEMA in 10 mM PBS (final volume 1 ml) at 37ºC. Spectrophotometric absorbance at 234 nm was monitored at 10-minute intervals for up to 360 minutes at 37ºC.

Assessment of Cholesterol Content

The amount of total cholesterol was determined using a cholesterol enzyme kit (Menarini Co., Italy) (Lee *et al*., 2002). LDL (200 μg protein/ml) was oxidized by $CuSO_4$ (20 μM) or AAPH (2 mM) in the presence or absence of FCBA/AEMA (μg/ml) for 24 hours. Samples were mixed with a working solution containing cholesterol esterase, cholesterol oxidase, and peroxidase to form quinoneimine at 37ºC for 10 minutes. The absorbance at 500 nm, which is proportional to the concentration of total cholesterol, was read.

Electrophoretic Mobility

The electrophoretic mobility of native or oxidized LDL was monitored by using agarose gel electrophoresis (Erl *et al*., 1998). LDL oxidation was carried out in the dark at 37ºC for 24 hours. The reaction solution consisted of 10 mM PBS containing $CuSO_4$ (20 μM) or AAPH (2 mM) at 37ºC, and 200 μg/ml LDL protein. The reaction was stopped at 4ºC. The LDL particles (approximately 2 μg LDL protein) were subjected to gel electrophoresis in 1% agarose gels with TAE buffer (pH 8), at 100 V for 40 minutes, dried at 65ºC, stained with Sudan Black B and evaluated for relative electrophoretic mobility (REM) and migration distance (cm) compared to native LDL.

Electrophoresis of Apo B Fragmentation

LDL oxidation was carried out in the dark at 37ºC for 24 hours. The reaction solution consisted of 10 mM PBS containing $CuSO_4$ (final concentration 20 μM) or AAPH (final concentration 2 mM) at 37ºC, and 200 μg/ml LDL protein. The LDL particles (approximately 6 μg LDL protein), after the oxidation with or without FCBA or AEMA, were denatured with 3% SDS, 10% glycerol, and 5% 2-mercaptoethanol at 95ºC for 5 minutes. SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 3-15% gradient) was performed to detect the Apo B fragmentation. The electrophoresis was conducted at 80 (200) V for 180 minutes; the gel then stained with Coomassie Brilliant Blue R250 and dried (Miura *et al*., 1994).

LDL Oxidation and HUVEC Cytotoxicity

The human umbilical vein endothelial cells (HUVECs) were prepared essentially as previously described (Yang *et al*., 1994) and grown in M-199 containing 20% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml) in a 5% CO_2 humidified incubator at 37°C. After reaching confluence, the primary cultured cells were detached using trypsin-EDTA and subcultured in six-well tissue culture plates with 20% FBS at a density of 5×10^5 cells. All experiments were carried out using single-passage cells a minimum of four days after the passage. After confluence, the original medium was removed and the cells washed three times with Ca^{2+} and Mg^{2+}-free Hank's Balanced Salt Solution. M-199 medium containing LDL

(200 μg protein/ml) incubated with 20 μM CuSO4 was added to the culture cluster. FCBA (50 μg/ml) or AEMA (200 μg/ml) was added to the incubation mixture to test its capacity to block LDL oxidation and HUVEC cytotoxicity under these conditions. The oxidation reaction was stopped at 4ºC. The cell number and morphology of each confluent monolayer were checked before and after the 24-hour treatment with FCBA or AEMA using trypan blue exclusion and phase contrast microscopy, respectively. The culture medium was collected and centrifuged at 1000 rpm for 10 minutes to remove the endothelial cells, pre- and post-incubation measurements of LDL TBARS formation (nmol/ml medium) and electrophoretic mobility (cm), as described above. Cholesterol level was determined in isolated cells extracted with chloroform and dried in nitrogen. The cellular cholesterol content was measured as described above. Protein concentration in the final LDL and endothelial cell preparations was determined using a Bio-Rad kit (Richmond, CA, USA), using bovine serum albumin as a standard.

Statistical Analysis

Mean data values are presented with standard error of the mean (mean ± SEM). Analysis of variance (ANOVA) followed by Dunnett's test for pairwise comparison was used for statistical validation of the data, with significance defined as $p < 0.05$.

Results

To evaluate the antioxidant effects of FCBA and AEMA on LDL oxidation, the LDL was incubated with increasing concentrations of FCBA and AEMA, and submitted to oxidation with metal ion-dependent $CuSO_4$ or metal ion-independent AAPH. These degenerative changes depend on a common initiating step, the peroxidation of polyunsaturated fatty acid (PUFA) components, which leads to extensive fragmentation into degradation products (Esterbauer *et al.*, 1989). In this study, the effect of FCBA or AEMA on the degree of LDL oxidation was analyzed using TBARS formation, conjugated diene production, cholesterol degradation, electrophoretic mobility, and Apo B fragmentation.

TBARS Formation

It has been reported that oxidative LDL generates peroxy radicals, leading to a chain reaction that causes an increase in lipid peroxides, as reflected in TBARS level (Esterbauer *et al*., 1989). Table 1 shows that incubation of LDL (50 μg protein/ml) with 10 μM $CuSO_4$ or 1 mM AAPH caused a marked TBARS increase at 6–24 hours. When FCBA (6–50 μg/ml) or AEMA (25–200 μg/ml) were added to the incubation, TBARS formation in the $CuSO_4$- or AAPH-induced LDL was inhibited in a time- and dose-dependent manner. The antioxidative effects of Trolox (at concentrations of 7.5 and 15 μg/ml) on LDL TBARS formation under these conditions were used as a positive control.

Table 1. Inhibitory Effects of FCBA/AEMA on CuSO4- or AAPH-Induced LDL Oxidation, as Measured by TBARS Formation

	TBARS (nmol MDA/mg protein)		
Experimental Condition	**6 Hours**	**12 Hours**	**24 Hours**
Control	1.36 ± 0.63[#]	0.88 ± 0.08[#]	0.88 ± 0.11[#]
LDL + $CuSO_4$	42.78 ± 0.64[*]	44.85 ± 1.11[*]	47.89 ± 0.69[*]
+6 μg/ml FCBA	40.31 ± 1.50[*]	44.31 ± 1.95[*]	45.90 ± 0.89[*]
+12 μg/ml FCBA	19.92 ± 1.15[*,#]	43.23 ± 4.71[*]	44.26 ± 2.03[*]
+25 μg/ml FCBA	1.29 ± 0.11[#]	3.44 ± 0.16[#]	3.51 ± 1.39[#]
+50 μg/ml FCBA	0.20 ± 0.02[#]	1.77 ± 0.22[#]	1.16 ± 0.18[#]
+25 μg/ml AEMA	42.27 ± 1.44[*]	46.83 ± 3.31[*]	47.27 ± 0.82[*]
+50 μg/ml AEMA	22.70 ± 1.02[*,#]	33.54 ± 1.05[*]	42.52 ± 1.60[*]
+100 μg/ml AEMA	1.84 ± 0.04[#]	0.73 ± 0.18[#]	36.90 ± 3.41[*]
+200 μg/ml AEMA	1.50 ± 0.23[#]	1.25 ± 0.46[#]	23.93 ± 1.68[*,#]
+7.5 μg/ml Trolox	2.49 ± 0.61[#]	36.80 ± 2.34[*]	40.38 ± 0.08[*]
+15 μg/ml Trolox	0.37 ± 0.05[#]	35.32 ± 3.68[*]	40.38 ± 0.08[*]
Control	1.06 ± 0.33[#]	0.19 ± 0.35[#]	1.35 ± 0.26[#]
LDL + AAPH	23.48 ± 3.04[*]	26.58 ± 2.45[*]	28.19 ± 2.69[*]
+6 μg/ml FCBA	19.76 ± 1.57[*]	22.10 ± 1.19[*]	24.11 ± 1.75[*]
+12 μg/ml FCBA	16.18 ± 0.99[*]	19.61 ± 2.16[*]	22.53 ± 2.76[*]
+25 μg/ml FCBA	5.41 ± 0.58[*,#]	13.58 ± 1.08[*,#]	19.74 ± 0.75[*,#]
+50 μg/ml FCBA	1.92 ± 0.10[#]	1.38 ± 0.42[#]	2.27 ± 0.91[#]
+25 μg/ml AEMA	21.66 ± 2.73[#]	24.68 ± 1.52[*]	37.05 ± 1.81[*]
+50 μg/ml AEMA	17.10 ± 2.26[*]	22.88 ± 2.51[*]	24.82 ± 1.62[*]
+100 μg/ml AEMA	5.02 ± 1.43[*]	10.05 ± 0.39[*,#]	17.33 ± 1.02[*,#]
+200 μg/ml AEMA	2.00 ± 0.23[#]	4.66 ± 1.60[#]	6.94 ± 1.11[*,#]
+7.5 μg/ml Trolox	0.21 ± 0.06[#]	0.27 ± 0.11[#]	10.79 ± 0.56[*,#]
+15 μg/ml Trolox	0.51 ± 0.08[#]	0.63 ± 0.11[#]	1.84 ± 1.55[#]

LDL (50 μg protein/ml) was oxidized by CuSO4 (10 μM) or AAPH (1 mM) in the presence or absence of FCBA/AEMA at the indicated concentrations for 6, 12 and 24 hours. MDA production was measured by the TBARS assay as described in the "Materials and Methods" section. Values are expressed as mean ± SEM of three to six experiments; [*]significant difference from control group (LDL; $p < 0.05$); [#]significant difference from $CuSO_4$- or AAPH-oxidized LDL group ($p < 0.05$).

Conjugated Diene Formation and Cholesterol Degradation

Large quantities of lipid peroxide are accompanied by rearrangement of the fatty acid double bond, yielding conjugated dienes, and induce cholesterol degradation (Esterbauer *et al*., 1989). The effects of FCBA or AEMA on LDL conjugated diene formation were examined by incubating LDL (50 μg protein/ml) with 10 μM $CuSO_4$ or 1 mM AAPH. As shown in Fig. 1, the addition of FCBA (0.4–6.4 μg/ml) or AEMA (1.6–25 μg/ml) to the reaction mixture resulted in prolongation of lag time. In this study, the effects of FCBA and AEMA on LDL cholesterol degradation were also examined by incubating the LDL (200 μg protein/ml) with 20 μM $CuSO_4$ or 2 mM AAPH. Figure 2 shows the amount of total cholesterol (measured enzymatically) when LDL was oxidized in the absence or presence of FCBA (6–100 μg/ml) or AEMA (25–400 μg/ml) for 24 hours. The decrease

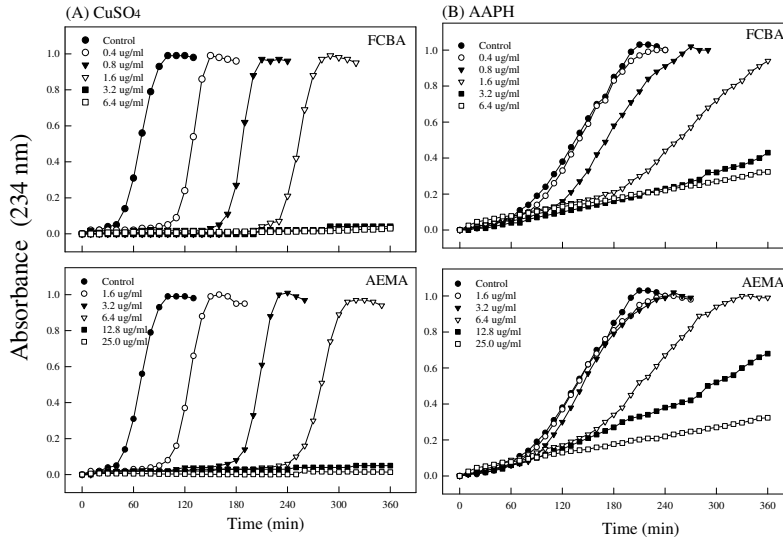

Figure 1. Inhibitory effects of FCBA/AEMA on (A) $CuSO_4$- or (B) AAPH-induced LDL oxidation as measured by conjugated diene formation. LDL (50 μg protein/ml) was oxidized by $CuSO_4$ (10 μM) or AAPH (1 mM) in the presence or absence (control) of FCBA/AEMA at the indicated concentrations for 360 minutes. The formation of conjugated dienes was monitored continuously at 10-minute intervals based on the absorbance at 234 nm, with the results expressed as absolute absorbance. This representative experiment was replicated with LDL from different donors with similar results observed.

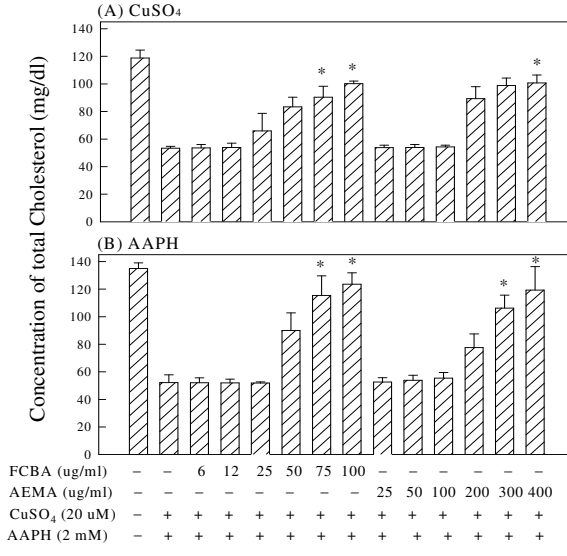

Figure 2. Inhibitory effects of FCBA/AEMA on (A) $CuSO_4$- or (B) AAPH-induced LDL oxidation as measured by cholesterol degradation. LDL (200 μg protein/ml) was oxidized by $CuSO_4$ (20 μM) or AAPH (2 mM) in the presence or absence of FCBA/AEMA at the indicated concentrations for 24 hours. The total cholesterol contents were determined as described in the "Materials and Methods" section. Values are expressed as mean ± SEM (three to six experiments); *significant difference compared to the $CuSO_4$ or AAPH groups ($p < 0.05$).

of total cholesterol in the $CuSO_4$- or AAPH-oxidized LDL was remarkably inhibited in the presence of either FCBA or AEMA, in a dose-dependent manner.

Electrophoretic Mobility

Oxidation of LDL causes an alteration in electrophoretic mobility of the lipoprotein due to an increase in the net charge of the protein (Steinbrecher *et al*., 1987). Hence, estimation of relative electrophoretic mobility is a further measure of LDL oxidation. The inhibitory effects of FCBA and AEMA on increased LDL electrophoretic mobility are shown in Fig. 3. Incubation of LDL (200 μg protein/ml) for 24 hours with 20 μM $CuSO_4$ or 2 mM AAPH at 37ºC resulted in oxidation modification of LDL, as evidenced by an increase in negative charge detected by agarose gels. When the LDL was oxidized in the presence of FCBA (6–100 μg/ml) or AEMA (25–400 μg/ml), however, the electrophoretic mobility of the LDL preparations was suppressed in a dose- dependent manner.

Apo B Fragmentation

Fatty acid fragmentation occurs consequent to the propagation reactions leading to the formation of highly reactive intermediates, such as aldehydes and ketones, which can then complex with the adjacent apo B to induce apo B fragmentation (Witztum, 1991).

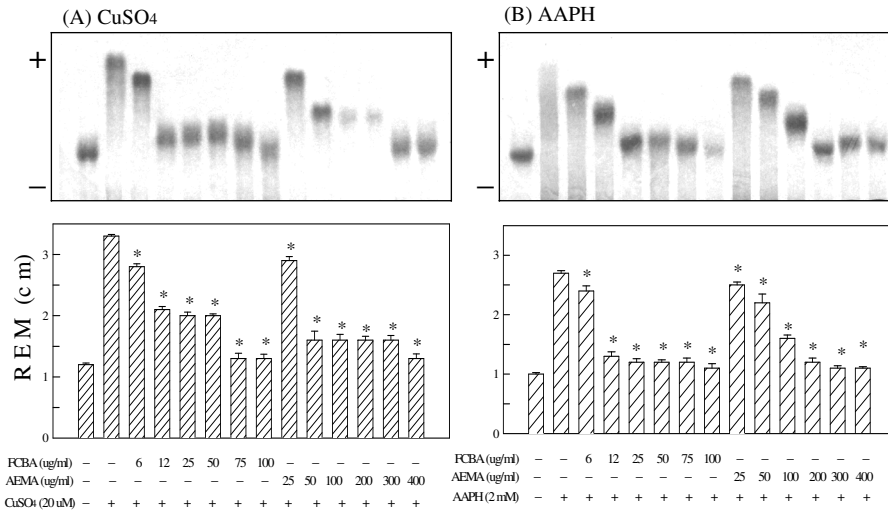

Figure 3. Inhibitory effects of FCBA/AEMA on (A) $CuSO_4$- or (B) AAPH-induced LDL oxidation as monitored by electrophoretic mobility. LDL (200 μg protein/ml) was oxidized by $CuSO_4$ (20 μM) or AAPH (2 mM) in the presence or absence of FCBA/AEMA at the indicated concentrations for 24 hours. LDL protein (2 μg) was applied to agarose gels (1%) and stained with Sudan Black B, evaluated for relative electrophoretic mobility (REM), and migration distance (cm) was measured. This representative experiment was replicated using LDL from different donors with similar results.

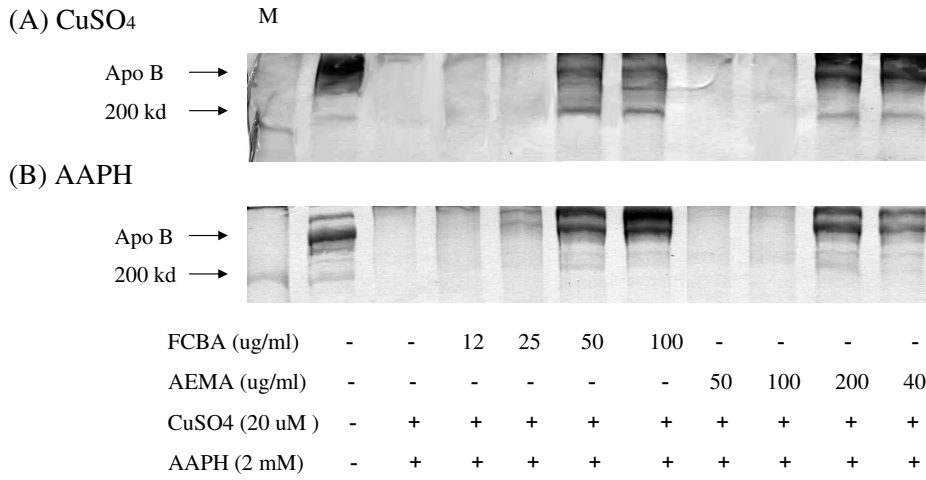

Figure 4. Inhibitory effects of FCBA/AEMA on (A) $CuSO_4$- or (B) AAPH-mediated Apo B fragmentation in LDL. LDL (200 μg protein/ml) was oxidized by $CuSO_4$ (20 μM) or AAPH (2 mM) in the presence or absence of FCBA/AEMA at the indicated concentrations for 24 hours. LDL protein (6 μg) was applied to SDS-PAGE (3%–15%) and stained with Coomassie Brilliant Blue R250. This representative experiment was replicated using LDL from different donors with similar results. M: Molecular weight markers.

The inhibitory effects of FCBA (12–100 μg/ml) and AEMA (50–400 μg/ml) on Apo B fragmentation were studied on 3%–15% SDS-PAGE (Fig. 4). The Apo B band was observed on native LDL that had been incubated without $CuSO_4$ or AAPH at 37ºC, however, the band disappeared after a 24-hour incubation of $CuSO_4$ (20 μM) or AAPH (2 mM). Fragmentation of LDL Apo B was inhibited in the presence of FCBA or AEMA.

LDL Oxidation and HUVEC Cytotoxicity

Oxidative LDL can be taken up by the cells, leading to several changes in cellular behavior, and finally, causing cytotoxic effects (Berliner and Heinecke, 1996). In this study, we have addressed the issue of the efficacy of FCBA and AEMA treatment with respect to protection of endothelial cells from the damaging effects of oxidized LDL. The LDL (200 μg protein/ml) was oxidized for 30 minutes with 20 μM $CuSO_4$ at 37ºC. Subsequently, HUVECs were treated with the $CuSO_4$-oxidized LDL in the absence or presence of FCBA (50 μg/ml) or AEMA (200 μg/ml) for 24 hours. In this study, exposure of the HUVECs to 20 μM $CuSO_4$-oxidized LDL caused significant cell damage and loss of cell viability (Fig. 5). Both the FCBA and AEMA protected the endothelial cells from oxidative damage and increased the cell viability of the $CuSO_4$-oxidized LDL. In isolation, 20 μM $CuSO_4$ was not obviously cytotoxic. As seen in Table 2, FCBA or AEMA was able to protect endothelial cells from the deleterious effects of $CuSO_4$-oxidized LDL, with efficacy measures including TBARS formation, relative electrophoretic mobility, and cellular cholesterol content.

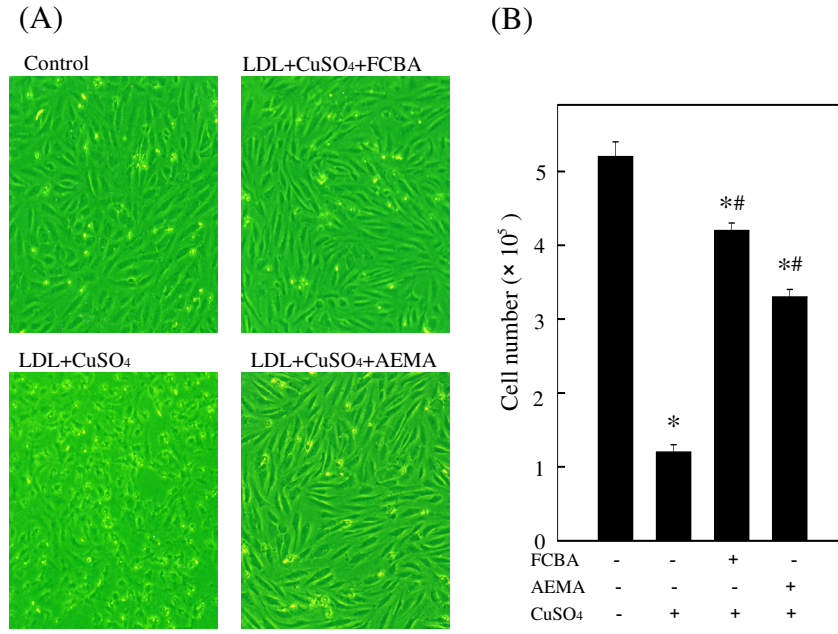

Figure 5. Cytoprotective effect of FCBA/AEMA against oxidized LDL-induced cytotoxicity in HVUECs. LDL (200 µg protein/ml) was oxidized for 30 minutes by 20 µM $CuSO_4$ at 37ºC, with HUVECs subsequently treated with $CuSO_4$-oxidized LDL in the presence or absence of FCBA (50 µg/ml) /AEMA (200 µg/ml) for 24 hours. Cell numbers in, and morphology of, the HUVEC monolayers were examined using Trypan Blue exclusion and phase-contrast microscopy. Values are expressed as mean ± SEM (three to six experiments); *significant difference compared to the control group (LDL; $p < 0.05$). #significant difference compared to the $CuSO_4$ or AAPH groups ($p < 0.05$).

Table 2. Effects of FCBA/AEMA on TBARS Formation, REM, and Cellular Cholesterol Content in $CuSO_4$-oxidized LDL-Treated HUVECs

	TBARS (nmol/ml medium)	REM (cm)	Cellular Cholesterol (µg/mg cell protein)
Control	1.8 ± 0.4	1.5 ± 0.1	17.4 ± 7.9
LDL + $CuSO_4$	30.1 ± 2.6*	3.2 ± 0.2*	193.1 ± 47.5*
LDL + $CuSO_4$ + FCBA	1.4 ± 0.1#	1.5 ± 0.1#	15.4 ± 1.2#
LDL+ $CuSO_4$ + AEMA	3.1 ± 0.3*,#	1.7 ± 0.1#	26.9 ± 4.2#

HUVECs were subsequently treated with $CuSO_4$-oxidized LDL in the absence or presence of FCBA (50 µg/ml)/ AEMA (200 µg/ml) for 24 hours. The culture medium was then collected and centrifuged at 1000 rpm for 10 minutes to remove the endothelial cells, followed by measurement of TBARS formation (nmol/ml medium) and electrophoretic mobility (cm), as described in the "Materials and Methods" section. Cholesterol level was determined from isolated endothelial cells extracted with chloroform and dried under nitrogen. The cellular cholesterol content (µg/mg protein) was measured using a cholesterol enzyme kit as described in the "Materials and Methods" section. Values are expressed as mean ± SEM (three to six experiments); *significant difference in comparison to the control group (LDL; $p < 0.05$); #significant difference from $CuSO_4$-oxidized LDL group ($p < 0.05$).

Discussion

It has been recognized that oxidative modification of LDL plays an important role in the initiation and progression of atherosclerosis. Cytotoxicity, chemotactic effects on monocytes, inhibition of macrophage motility, and uptake by the macrophage scavenger receptor result in stimulation of cholesterol accumulation and, hence, foam cell formation (Quinn *et al*., 1985). To prevent atherosclerosis and vascular disease, therefore, inhibition of the oxidative modification of LDL is important. In the present work, we conclude that *A. camphorata* (as determined for both the fermented culture broth and aqueous extract of its mycelia harvested from submerged cultures) does appear to possess antioxidant properties. We have shown that both FCBA and AEMA are able to inhibit oxidative modification of LDL induced by $CuSO_4$ and AAPH incubation, as measured by TBARS formation, conjugated diene production, cholesterol degradation, electrophoretic mobility, and Apo B fragmentation. It was also determined that the culture medium had low antioxidant ability relative to the FCBA or AEMA in submerged culture (data not shown), indicating that the antioxidant properties of *A. camphorata* must be derived from secondary metabolites of mycelia. The atherogenicity of the oxidized LDL also involves cytotoxicity against the arterial wall cells as well as proinflammatory and thrombotic properties (Yla-Herttuala *et al*., 1989; Berliner *et al.*, 1995; Aviram, 1996). In this study, incubation of the human endothelial cells in the presence of FCBA/AEMA and challenge with $CuSO_4$-induced oxidative LDL revealed that, in addition to antioxidant effects, *A. camphorata* was also able to provide vascular protection, effectively preventing the cells from the damaging effects of the oxidized LDL. The results suggest that *A. camphorata* inhibits the oxidative modification of LDL in both cell-free and endothelial-cell models. Since our results indicate that both FCBA and AEMA possess strong antioxidant effects, further investigation should seek to verify this effect in animal models.

Our results indicate that the inhibitory effects of *A. camphorata* on LDL oxidation were greater for the $CuSO_4$-treated samples than for the AAPH analogs. This suggests that *A. camphorata* may have strong chelating activity, which may complement its other antioxidant properties (Lin *et al*., 2001) and explain the differential effects obtained with FCBA/AEMA when in the presence AAPH or $CuSO_4$.

Numerous studies have demonstrated a range of pharmacological properties, associated with antioxidant, anti-inflammatory, anti-mutagenic, and anti-carcinogenic activities, in both medicinal and edible mushrooms (Huang and Ferraro, 1992; Rao and Gurfinkel, 2000; Liu *et al*., 1997; Zhu *et al.*, 1999; Wasser and Weis, 1999; Ma *et al.*, 1991). Compounds isolated from *A. camphorata* include polysaccharides, ergostan-type triterpenoids, a sesquiterpene, and phenyl and biphenyl derivatives (Huang, 2000; Dicorleto and Chisolm, 1986; Chen and Yang, 1995). The yields of total polysaccharides, polyphenols, and crude triterpenoids were about 23%, 67 mg/g, and 47 mg/g for FCBA, 12%, 71 mg/g, and not detectable for AEMA, respectively. Evaluation using different antioxidant tests has demonstrated an association between the antioxidant activity of *A. camphorata* and its polysaccharide, polyphenol, and triterpenoid contents (Huang and Ferraro, 1992). In contrast, no polysaccharides, total polyphenols, or crude triterpenoids were detected in the dry matter of the culture medium

(Huang and Ferraro, 1992). It seems reasonable to suggest, therefore, that *A. camphorata* metabolizes the medium to release active components such as these during fermentation of the submerged culture. The results imply that higher contents of natural polyphenols, triterpenoids, and polysaccharides of *A. camphorata* extracts possibly act as preventive agents with respect to inhibition of oxidative modification of LDL. However, identification and characterization of possible compounds which may account for the antioxidant activity of *A. camphorata* requires further study.

In conclusion, *A. camphorata* supplementation reduced both $CuSO_4$- or AAPH-induced oxidative modification of LDL in our study. Additionally, our *A. camphorata* preparations were able to protect endothelial cells from the damaging effects of oxidized LDL. Based on these findings, it seems reasonable to suggest that the antioxidant properties of *A. camphorata* may also provide effective protection from atherosclerosis. Further investigation of protection *in vivo* is warranted to confirm these promising results, however.

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