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Garlic Compounds Protect Vascular Endothelial Cells from Oxidized Low Density Lipoprotein-induced Injury

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Abstract

Oxidation of low density lipoprotein (LDL) has been recognized as playing an important role in the initiation and progression of atherosclerosis. In this study, the effects of aged garlic extract and one of its major compounds, *S*-allylcysteine, on oxidized LDL-induced cell injury were studied.

Pulmonary artery endothelial cells were pre-incubated with the garlic extract (1, 2·5 and 5 mg mL^{-1}) or *S*-allylcysteine (0·1, 1, 10 and 20 mM) at 37°C and 5% CO_2 for 24 h, washed, and then exposed to 0·1 mg mL^{-1} oxidized LDL for 24 h. Lactate dehydrogenase release as an index of membrane damage, methylthiazol tetrazolium assay for cell viability and thiobarbituric acid reactive substances indicating lipid peroxidation were determined. Preincubation of endothelial cells with the extract or *S*-allylcysteine significantly prevented membrane damage, loss of cell viability and lipid peroxidation.

The data indicate that these compounds can protect vascular endothelial cells from injury caused by oxidized LDL, and suggest that they may be useful for prevention of atherosclerosis.

Oxidation of low density lipoprotein (LDL) has been recognized as playing an important role in the initiation and progression of atherosclerosis (Steinberg et al 1989; Cox & Cohen 1996). LDL can be oxidized by incubation of endothelial cells with a transition metal such as copper or iron (Henriksen et al 1981). Oxidized LDL promotes vascular dysfunction by exerting direct cytotoxicity toward endothelial cells (Kuzuya et al 1991), by increasing chemotactic properties in monocytes (Quinn et al 1987) and by transforming macrophages to foam cells via scavenger-receptors (Henriksen et al 1981); all these events are recognized to contribute to atherosclerosis. We have reported earlier that an aged garlic extract (AGE) and one of its major constituents, *S*-allylcysteine (SAC), protected vascular endothelial cells from hydrogen peroxide (H_2O_2)-induced oxidant injury (Yamasaki et al 1994). More recently, we also reported that garlic compounds inhibited Cu^{2+}-induced LDL oxidation (Ide & Lau 1997). In the present study, we determined the effects of AGE and SAC on oxidized LDL-induced injury in vascular endothelial cells.

Materials and Methods

Chemicals

Aged garlic extract (AGE) and *S*-allylcysteine (CH_2=CH–CH_2–SCH_2–$CHNH_2$–COOH) were provided by Wakunaga Pharmaceutical Co. Ltd, Osaka, Japan. Human low density lipoprotein (LDL), Hanks' balanced salt solution (HBSS), 2-thiobarbituric acid (TBA), sodium dodecyl sulphate sodium salt (SDS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Triton X-100, tetraethoxypropane and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co., St Louis, MO. *n*-Butanol was from Fisher Scientific Co., Fair Lawn, NJ. CytoTox96 Nonradioactive Cytotoxicity Assay Kit was supplied by Promega Co., Madison, WI. Eagle's minimum essential medium (EMEM), trypsin-EDTA solution, and penicillin–streptomycin solution were from Mediatech Co., Washington, DC. Bovine calf serum (BCS) was obtained from HyClone Laboratories, Logan, UT.

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Cell line

Bovine pulmonary artery endothelial cells were obtained from American Type Culture Collection, Rockville, MD. Cells were grown in EMEM supplemented with 20% BCS, 200 units mL^{-1} penicillin, and 0·2 mg mL^{-1} streptomycin. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 3–4 days before being used for experiments.

Preparation of oxidized low density lipoprotein

LDL was dialysed at 4°C for 48 h against 500 vols PBS to remove EDTA. For preparation of oxidized LDL, LDL (5 mg mL^{-1}) was incubated with 20 μM $CuSO_4$ at 37°C for 24 h and then dialysed at 4°C for 48 h against 500 vols PBS to remove Cu^{2+} ions (Kuzuya et al 1991). Protein contents were determined (Lowry et al 1951). Oxidized LDL was confirmed with agarose gel electrophoresis as described previously (Nobel 1968).

Lactate dehydrogenase (LDH) release

Endothelial cells (8×10^4 per well) in 24-well plates were pre-incubated with different concentrations of AGE (1, 2·5, and 5 mg mL^{-1}) or SAC (0·1, 1, 10, and 20 mM) for 24 h, washed with HBSS, and then incubated with 0·1 mg mL^{-1} oxidized LDL in HBSS for 24 h. The supernatant was collected from each well and stored at 4°C. Cell monolayers were treated with lysing solution (0·8% Triton X-100) for 30 min at room temperature to lyse the cell membranes, and the lysate was collected. LDH activity was measured in both the supernatant and the cell lysate fractions by using CytoTox96 Nonradioactive

Cytotoxicity Assay Kit following the manufacturer's instructions. The assay is based on the conversion of a tetrazolium salt into a red formazan product. The intensity of colour is proportional to LDH activity. The absorbance was determined at 492 nm in an ELISA reader (400 AT EIA, Whittaker Bioproducts, Walkersville, MD). The percent of LDH released from the cells was determined using the formula: Release (%) = LDH activity in supernatant/(LDH activity in supernatant + LDH activity in cell lysate).

MTT assay for cell viability

Endothelial cells (8×10^3/well) in 96-well plates were pre-incubated with different concentrations of AGE or SAC for 24 h, washed with HBSS, and then incubated with 0·1 mg mL^{-1} oxidized LDL in HBSS for 24 h. Cells were washed with HBSS, and then MTT (0·4 mg mL^{-1}, 100 (μL) was added to each well. After incubation for 5 h at 37°C, 100 μL of 10% SDS was added to dissolve the formazan crystals. Plates were incubated at 37°C overnight, and the absorbance was measured at 620 nm using the ELISA reader.

Lipid peroxidation

Cells (8×10^4/well) in 24-well plates were pre-incubated with different concentrations of AGE or SAC for 24 h, washed with HBSS, and then incubated with 0·1 mg mL^{-1} oxidized LDL in HBSS for 24h. The supernatant in the wells was collected, and the extent of lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) as described previously (Wey et al 1993). Trichloroacetic acid (12·5%, 0·2 mL) was added to a sample of the supernatant. TBA reagent (0·4 mL 0·67% TBA and 1 mM EDTA) was added. The reaction mixture was heated at 95°C for 20 min. After cooling with tap water, 3 mL *n*-butanol was added, and the mixture was shaken vigorously for 30 s. After centrifugation at 2000 rev min^{-1} for 10 min, the *n*-butanol layer was removed and measured with excitation of 515 nm and emission of 553 nm, using LS-3 Fluorescence Spectrophotometer (Perkin-Elmer, Norwalk, CT). The value of fluorescence was calculated by comparing with standards prepared from tetraethoxypropane.

Statistical analysis

Data were analysed with one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for significant difference. Results were expressed as means±s.e. A P value of less than 0·05 was considered significant. All statistical procedures were performed with Statgraphics software version 5·0 (STSC, Inc., Rockville, MD).

Results

LDH is an intracellular enzyme that leaks into the culture medium when cell membranes are damaged. Table 1 shows the effects of AGE and SAC on LDH release from endothelial cells. Exposure to oxidized LDL caused a four-fold increase in LDH release compared with unexposed cells, indicating that oxidized LDL induced cell damage. Pretreatment with AGE at 1, 2·5 and 5 mg mL^{-1} inhibited LDH release by 11·3%, 35·1% and 50·5%, respectively. SAC exhibited a dose-dependent inhibition of LDH release ranging from 10·4 to 91·0%, and the inhibition observed at the three higher dosages was statistically significant.

The MTT assay was used to measure cell viability. MTT is a pale yellow substrate that produces a dark blue formazan product when incubated with living cells. The MTT ring is cleaved by mitochondrial dehydrogenase, and this reaction occurs only in living cells. When endothelial cells were treated with oxidized LDL for 24 h, there was a significant decrease in cell viability compared with untreated cells. Pretreatment with AGE or SAC resulted in a dose-dependent increase in cell viability in oxidized LDL-treated cells. The increase was significant with AGE at 2·5 and 5 mg mL^{-1}, and with SAC at all four concentrations (Table 2).

The effects of AGE and SAC on lipid peroxidation were determined by measuring TBARS, products of lipid peroxidation. When endothelial cells were incubated with oxidized LDL for 24 h, there was a 14-fold increase in TBARS. Pretreatment of cells with AGE or SAC significantly inhibited lipid peroxidation in Ox-LDL-treated cells as evidenced by decreases in TBARS (Table 3).

Discussion

It has been known for more than two decades that an elevated plasma level of LDL is associated with the development of atherosclerosis (Kannel et al 1971). More recently, oxidation of LDL has been recognized as an important factor to many atherosclerogenic actions ascribed to LDL (Steinberg et al 1989; Cox & Cohen 1996).

Table 1. Effects of aged garlic extract (AGE) and *S*-allylcysteine (SAC) on oxidized LDL-induced lactate dehydrogenase (LDH) release from bovine pulmonary artery endothelial cells.

Pretreatment[a]	Oxidized LDL[b] (0·1 mg mL^{-1})	LDH release[c] (% of total)	Inhibition (%)
None	–	7·30±0·28	–
None	+	29·50±0·99	–
AGE (1 mg mL^{-1})	+	27·00±0·52	11·3
AGE (2·5 mg mL^{-1})	+	21·70±0·93*	35·1
AGE (5 mg mL^{-1})	+	18·30±0·39*	50·5
SAC (0·1 mM)	+	27·20±0·25	10·4
SAC (1 mM)	+	23·70±1·07*	26·1
SAC (10 mM)	+	13·90±0·74*	70·3
SAC (20 mM)	+	9·30±0·28*	91·0

[a]Vascular endothelial cells were incubated with AGE or SAC for 24 h, and washed before exposure to oxidized LDL for 24 h. [b]Treatment with oxidized LDL (+); not treated with oxidized LDL (−). [c]Values are means±s.e. of triplicate samples. *Significantly different from control exposed to oxidized LDL without AGE or SAC pretreatment ($P < 0·05$).

Table 2. Effects of aged garlic extract (AGE) and *S*-allylcysteine (SAC) on oxidized LDL-induced cell damage.

Pretreatment[a]	Oxidized LDL[b] (0·1 mg mL^{-1})	MTT absorbance[c] (620 nm)	Cell viability (%)
None	−	0·137±0·010	100
None	+	0·056±0·002	40·9
AGE (1 mg mL^{-1})	+	0·061± 0·004	44·5
AGE (2·5 mg mL^{-1})	+	0·083±0·002*	60·6
AGE (5 mg mL^{-1})	+	0·108±0·005*	78·8
SAC (0·1 mM)	+	0·072±0·003*	52·6
SAC (1 mM)	+	0·081±0·006*	59·1
SAC (10 mM)	+	0·100±0·001*	73·0
SAC (20 mM)	+	0·114±0·003*	83·2

[a]Vascular endothelial cells were incubated with AGE or SAC for 24 h, and washed before exposure to oxidized LDL for 24 h. [b]Treatment with oxidized LDL (+); not treated with oxidized LDL (−). [c]Values are means ± s.e. of triplicate samples. *Significantly different from control exposed to oxidized LDL without AGE or SAC pretreatment ($P < 0·05$).

Table 3. Effects of aged garlic extract (AGE) and *S*-allylcysteine (SAC) on oxidized LDL-induced lipid peroxidation.

Pretreatment[a]	Oxidized LDL[b] (0·1 mg mL^{-1})	TBARS[c] (pmol)	Inhibition (%)
None	−	4·30±0·35	−
None	+	61·90±1·22	−
AGE (1 mg mL^{-1})	+	27·10±1·38*	60·4
AGE (2·5 mg mL^{-1})	+	35·10±1·71*	46·5
AGE (5 mg mL^{-1})	+	25·00±0·80*	64·1
SAC (0·1 mM)	+	47·80±2·30*	24·5
SAC 1 (mM)	+	42·20±2·09*	34·2
SAC 10 (mM)	+	42·10±1·74*	34·4
SAC 20 (mM)	+	29·20±2·41*	56·8

[a]Vascular endothelial cells were incubated with AGE or SAC for 24 h, and washed before exposure to oxidized LDL for 24 h. [b]Treatment with oxidized LDL (+); not treated with oxidized LDL (−). [c]Thiobarbituric acid reactive substance (TBARS) values are means ± s.e. of triplicate samples. *Significantly different from control exposed to oxidized LDL without AGE or SAC pretreatment ($P < 0·05$).

In the present study, we used a model of oxidant injury induced by oxidized LDL to investigate the antioxidant effect of AGE and one of its major compounds, SAC, on pulmonary artery endothelial cells. We demonstrated that Ox-LDL caused significant cell damage, as evidenced by the increase in LDH release. LDH is an intracellular enzyme that leaks from cells when their membranes are damaged. Our data indicated that pre-incubation with AGE or SAC significantly inhibited the increase in LDH release induced by oxidized LDL, showing the protective effects of these compounds on cell membranes. The MTT assay was used to monitor cell viability. Since MTT is cleaved only in active mitochondria (Mosmann 1983), oxidized LDL-induced decrease of cell viability measured by the MTT-assay indicates that Ox-LDL may have damaged the mitochondria. Pretreatment of endothelial cells with AGE or SAC resulted in a concentration-dependent increase in cell viability, suggesting a protective effect on the mitochondria of endothelial cells. To elucidate the mechanism of cell injury, TBARS–products of lipid peroxidation–were measured in oxidized LDL-stressed cells. Oxidized LDL caused a significant increase in TBARS. Pretreatment of cells with AGE or SAC significantly inhibited TBARS formation, indicating protection against lipid peroxidation.

We previously demonstrated that AGE lowers blood cholesterol and triglycerides in human subjects (Lau et al 1987). We have also reported that AGE and SAC protect vascular endothelial cells from H_2O_2-induced cell injury (Yamasaki et al 1994). More recently, we reported that AGE and several of its constituents inhibit Cu^{2+}-induced LDL oxidation, showing their extensive antioxidant activities (Ide & Lau 1997).

In conclusion, pretreatment of vascular endothelial cells with garlic compounds prevented membrane damage, loss of cell viability and lipid peroxidation. The data indicate that these compounds can protect vascular endothelial cells from dysfunction induced by oxidized LDL, and suggest that they may be useful in the prevention of atheriosclerosis.

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