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RESEARCH PAPER

S-Allylcysteine Attenuates Oxidative Stress in Endothelial Cells

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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. We recently reported that S*-allylcysteine (SAC), one of the major compounds in the aged garlic extract (AGE), inhibited LDL oxidation and minimized oxidized LDL–induced cell injury. In this study, the antioxidant effects of SAC were further determined using several in vitro assay systems. Pulmonary artery endothelial cells (PAECs) were preincubated with SAC at 37°C and 5% CO_2 for 24 hr, washed, and then exposed to 0.1 mg/ml oxidized LDL for 24 hr. Lactate dehydrogenase (LDH) release, as an index of membrane injury, and intracellular glutathione (GSH) levels were determined. Oxidized LDL caused an increase of LDH release and depletion of GSH. Pretreatment with SAC prevented these changes. Peroxides were measured directly in 24-well plates using a fluorometric assay. SAC dose-dependently inhibited oxidized LDL-induced release of peroxides in PAEC. In a cell-free system, SAC was shown to scavenge hydrogen peroxide. Our data demonstrate that SAC can protect endothelial cells from oxidized LDL-induced injury by removing peroxides and preventing the intracellular GSH depletion and suggest that this compound may be useful for the prevention of atherosclerosis.*

INTRODUCTION

Oxidation of low-density lipoprotein (LDL) is recognized as playing an important role in the initiation and progression of atherosclerosis (1,2). LDL can be oxidized by incubation of endothelial cells (3), smooth muscle cells (4), or monocytes/macrophages (5) with a transition metal such as copper or iron. Oxidized LDL (Ox-LDL) promotes vascular dysfunction by exerting direct cytotoxicity toward endothelial cells (6), by increasing mono-

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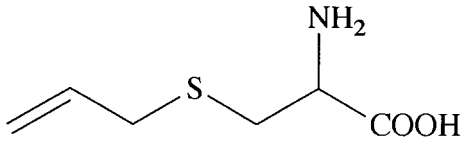

Figure 1. Structure of *S*-allylcysteine (SAC).

cyte chemotactic properties (7), and by transforming macrophages to foam cells via scavenger receptors (3); all these events are recognized as contributing to atherosclerosis.

S-Allylcysteine (SAC; Fig. 1) is one of the major water-soluble compounds in aged garlic extract (AGE) (8–10) and has been extensively studied by various investigators. SAC has been demonstrated to have antioxidant (11) and anticarcinogenic properties (12,13). Recent data from our laboratory have shown that SAC protects vascular endothelial cells from H_2O_2-induced injury (14). We have also demonstrated that this compound inhibits Cu^{2+}-induced LDL oxidation (15), minimizes oxidized LDL-induced cell injury (16), and inhibits nuclear factor kappa B activation in human T cells (17).

In this study, the antioxidant effects of SAC were determined using several in vitro assay systems. We now report that SAC can minimize depletion of intracellular glutathione (GSH) and the release of peroxides induced by Ox-LDL.

MATERIAL AND METHODS

Chemicals

S-Allylcysteine (SAC) was provided by Wakunaga Pharmaceutical Company, Limited Osaka, Japan. Human LDL, Hanks' balanced salt solution (HBSS), Triton X-100, trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), ethylenediamine tetraacetic acid (EDTA), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and phosphate buffered saline (PBS) were purchased from Sigma Chemical Company (St. Louis, MO). Horseradish peroxidase and 2,2′-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) were obtained from Boehringer Mannheim Company (Indianapolis, IN). Cupric sulfate ($CuSO_4$ $5H_2O$) was from J. T. Baker Chemical Company (Phillipsburg, NJ). 2′7′-Dichlorofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR). CytoTox96™ Nonradioactive Cytotoxicity Assay Kit was supplied by Promega Company (Madison, WI). Eagle's minimum essential medium (EMEM), trypsin-EDTA solution, and penicillin-streptomycin solution were from Mediatech Company (Washington, DC). Bovine calf serum (BCS) was obtained from HyClone Laboratories, Incorporated (Logan, UT).

Cell Line

Bovine pulmonary artery endothelial cells (PAECs) were obtained from American Type Culture Collection (Rockville, MD). The PAECs were grown in EMEM supplemented with 20% BCS, 200 U/ml penicillin, and 0.2 mg/ml streptomycin. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 3–4 days before experimental use. Viability of cells used throughout the experiment was always greater than 95% as determined by trypan blue exclusion.

Preparation of Oxidized LDL

The LDL was dialyzed at 4°C for 48 hr against 500 volumes of PBS to remove EDTA. For preparation of oxidized LDL, LDL (5 mg/ml) was incubated with 20 μM $CuSO_4$ at 37°C for 24 hr and then dialyzed at 4°C for 48 hr against 500 volumes of PBS to remove Cu^{2+} (6). Protein content was determined by the method of Lowry et al. (18). Presence of Ox-LDL was confirmed using agarose gel electrophoresis (19).

Lactate Dehydrogenase Release

The PAECs (8×10^4 cells/well) in 24-well plates were preincubated with 0.1, 1, and 10 mM of SAC for 24 hr, washed with HBSS, and then incubated with 0.1 mg/ml oxidized LDL in HBSS for 24 hr. The supernatant was collected from each well and stored at 4°C. Cell monolayers were treated with 0.2 ml of 0.8% Triton X-100 for 30 min at room temperature to disrupt cell membranes. The lysates were then collected. Lactate dehydrogenase (LDH) activity was measured in both the supernatant and the cell lysate fractions using the CytoTox96 Nonradioactive Cytotoxicity Assay Kit following the manufacturer's instruction. This assay is based on the conversion of a tetrazolium salt into a red formazan product. The intensity of color is proportional to LDH activity. Absorbance was determined at 492 nm with a 96-well plate enzyme-linked immunosorbent assay (ELISA) reader (400 AT EIA, Whittaker Bioproducts, Walkersville, MD). Percentage LDH released from the cells was determined using the formula

Percent release = LDH activity in supernatant/
(LDH activity in supernatant
+ LDH activity in cell lysate)

Determination of Intracellular GSH

Intracellular GSH was determined according to the method of Sedlak and Lindsay (20). The PAECs (4×10^6 cells) in 75-cm^2 flasks were preincubated with different concentrations of SAC for 24 hr, washed with HBSS, and then incubated with 0.1 mg/ml Ox-LDL in HBSS for 24 hr. After cell monolayers were washed with HBSS to remove Ox-LDL, they were treated with 0.2 ml of 0.8% Triton X-100 for 20 min at room temperature to lyse cell membranes. To measure the intracellular GSH, 0.2 ml of the lysates were added to 0.2 ml of 10% TCA, and the mixture was centrifuged at 15,000 rpm for 10 min at 4°C. To the supernatant was added 0.8 ml of 0.4 M Tris-HCl buffer (pH 8.9) and 20 μl of 10 mM DTNB-methanol solution. The absorbance was then measured at 412 nm using the Spectronic 2000 spectrophotometer (Bausch & Lomb, Rochester, NY). The GSH level was compared with that of endothelial cells without exposure to Ox-LDL and expressed as percentage of control.

Determination of Peroxides

Peroxides were measured by a fluorometric assay using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as a probe (21). DCFH-DA, a nonfluorescent compound, is deacetylated by viable cells to highly fluorescent 2′7′-dichlorofluorescein (DCF) by hydrogen peroxide and lipid peroxides. Confluent PAECs (8×10^4 cells/well) in 24-well plates were incubated with 0.2 ml of 0.1, 1, 10, and 20 mM of SAC in HBSS, 0.1 mg/ml Ox-LDL, 10 μl of 0.5 mM DCFH-DA. The fluorescence intensity (relative fluorescence unit) was measured at 485 nm excitation and 530 nm emission every 20 min for 3 hr and at the end of 24 hr using the 7620 Microplate Fluorometer (Cambridge Technology, Watertown, MA).

H_2O_2 Scavenging Assay

The scavenging effect of SAC on H_2O_2 was determined according to the method of Okamoto, Hayase, and Kato (22). One-tenth ml of 50 nM H_2O_2, 0.1 ml of different concentrations of SAC or HBSS, 0.6 ml of 10 U/ml peroxidase, and 0.6 ml of 0.1% ABTS were added to 1.8 ml of 0.1 M phosphate buffer (pH 6.0). The solution was then incubated at 37°C for 15 min. Absorbance at 414 nm was measured using the Spectronic 2000 spectrophotometer.

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for significant difference, and results were expressed as the mean ± SE. A p value of less than .05 was considered significant. All statistical procedures were performed with Statgraphics software version 5.0 (STSC, Inc., Rockville, MD).

RESULTS

Figure 2 shows the effects of SAC on LDH release (histogram) and GSH level (line graph) when cells were exposed to 0.1 mg/ml Ox-LDL. Ox-LDL caused an increase of 23% in LDH release and a decrease of 60% in intracellular GSH compared with cells not exposed to Ox-LDL. Pretreatment of cells with SAC resulted in a dose-dependent inhibition of LDH release and intracellular GSH depletion.

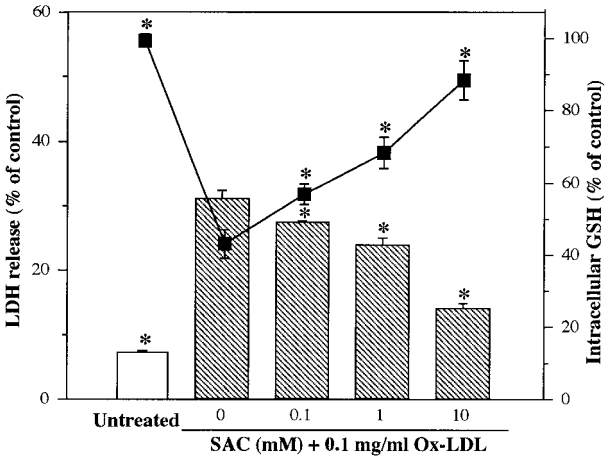

Figure 2. Effects of SAC on LDH release and intracellular GSH. Histogram depicts LDH release; bars represent means ± SE of triplicate samples. Line graph depicts intracellular GSH contents; each point represents mean ± SE of triplicate samples. Untreated = cells not exposed to Ox-LDL. *Significant difference ($P < .05$) compared with samples exposed to Ox-LDL but without SAC pretreatment.

Figure 3 shows the effects of SAC on release of peroxides induced by Ox-LDL. Exposure of PAECs to oxidized LDL resulted in a significant release of peroxides (Fig. 3A). Coincubation of SAC and Ox-LDL inhibited release of peroxides dose-dependently. Even after 24 hr of coincubation, SAC still exerted its inhibition at concentrations of 10 and 20 mM (Fig. 3B).

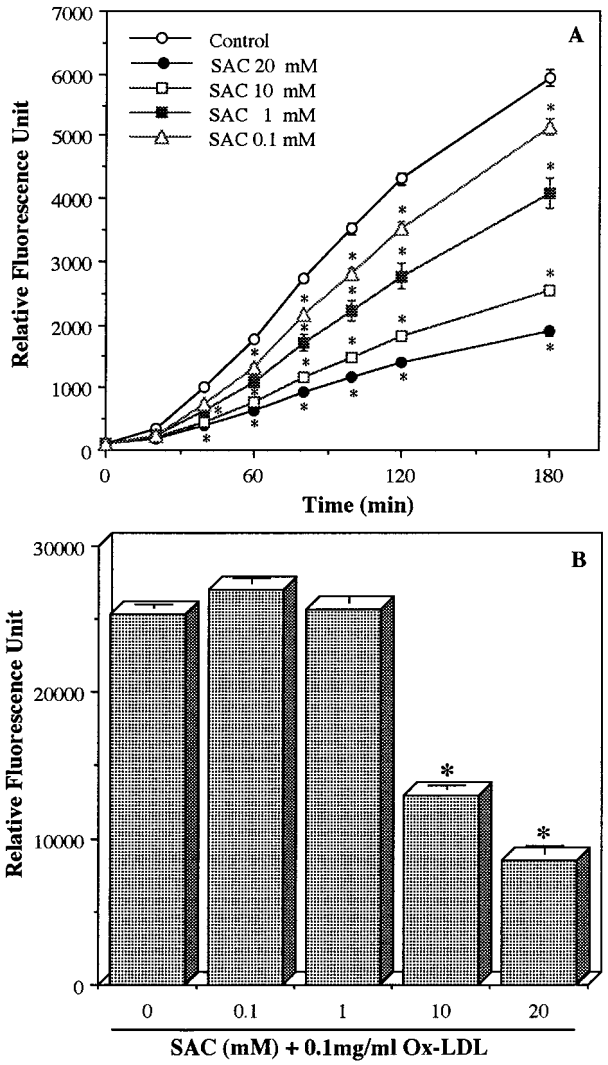

Figure 3. Effects of SAC on release of peroxides. PAECs were incubated with 0.1 mg/ml Ox-LDL and various concentrations of SAC. 2′7′-Dichlorofluorescein fluorescence, indicating release of peroxides, was monitored every 20 min for 3 hr (Fig. 3A). Release of peroxides was also measured at 24 hr (Fig. 3B). Data represent means ± SE of triplicate samples. *Significant difference ($P < .05$) compared with control exposed to Ox-LDL without SAC.

Table 1

Scavenging Effect of SAC on Hydrogen Peroxide

SAC (mM)	H_2O_2 (nmol)	Inhibition (%)
0	5.00	—
0.1	4.96 ± 0.03	0.8
1	4.26 ± 0.03[a]	14.8
10	0.92 ± 0.03[a]	81.6
20	1.04 ± 0.06[a]	79.2

Data represent mean ± SE of triplicate samples.
[a] Significant difference compared with samples without SAC ($p < .05$).

Table 1 shows the direct scavenging effect of SAC on H_2O_2. Decreases of H_2O_2 reflecting scavenging by SAC were noted, with significant activity observed at 1, 10, and 20 mM of SAC.

DISCUSSION

Low-density lipoprotein oxidation has been recognized as playing an important role in the initiation and progression of atherosclerosis (1,2). LDL has been shown to be oxidized by cultured cells such as macrophages and endothelial and smooth muscle cells with transition metals. Ox-LDL appears to initiate vascular dysfunction by directly promoting cell cytotoxicity. It can alter the composition and permeability of the endothelial barrier (23) and is thus cytotoxic for endothelial cells (24,25). We previously reported that garlic compounds inhibit Cu^{2+}-induced LDL oxidation (15) and protect vascular endothelial cells from Ox-LDL-induced cell injury (16). In the present study, LDH release was measured as an index of cell injury. LDH is an intracellular enzyme released into the medium on cell membrane damage. Ox-LDL caused an increase in LDH release, indicating cell injury. SAC exhibited a dose-dependent inhibition of Ox-LDL-induced LDH release (Fig. 2).

Ox-LDL has been shown to deplete intracellular GSH in cultured endothelial cells (25). Intracellular GSH depletion can lead to increased endothelial cell susceptibility to injury caused by Ox-LDL (24). GSH is the most abundant low molecular weight thiol compound in the cell and plays an important role in antioxidant defense and detoxification. GSH depletion compromises cell defenses against oxidative damage and may lead to cell death (26). Our data demonstrate that incubation of PAECs with Ox-LDL for 24 hr caused a 60% decrease of total GSH. Preincubation of PAECs with SAC prevented GSH depletion, suggesting that this compound can be a

potent protective agent against Ox-LDL-induced cytotoxicity.

Under oxidant-stressed conditions, peroxides such as H_2O_2 and lipid peroxides change cell function and interaction with surrounding cells. For instance, H_2O_2 serves as an important second messenger in the activation of the transcription factor, NF-$_\kappa$B, which is associated with expression of cell adhesion factors, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) (17,27). Furthermore, H_2O_2 damages cell membranes, reduces cell viability, and induces lipid peroxidation (14). H_2O_2 also yields hydroxyl radicals (· OH) by reacting with a transition metal such as Fe^{2+} or Cu^{2+}. Generation of these hydroxyl radicals results in DNA damage and lipid peroxidation, leading to cell dysfunction and death. Lipid peroxides in endothelial cells change the permeability of cell membranes and ion efflux. Lysophosphatidylcholine (LysoPC), which is a lipid peroxide composed of Ox-LDL, can be a trigger of inflammation that leads to inflammatory mediators such as reactive oxygen species, cytokines (e.g., tumor necrosis factor alpha [TNF-α] and interleukin-6 [IL-6]), arachidonic acid metabolites, and NO. In a time course study, SAC inhibited release of peroxides in endothelial cells dose-dependently (Fig. 3A) while still retaining its inhibitory effect 24 hr later. We also observed the direct scavenging effect of SAC on hydrogen peroxide. Our data thus suggest that SAC can protect endothelial cells from oxidant injury by removing the intracellular peroxides such as hydrogen peroxide and lipid peroxide.

SAC is one of the major water-soluble compounds derived from AGE. Its bioavailability has been well established in animals and has been shown to be evenly absorbed and distributed systemically (28). Although several beneficial antioxidant properties of SAC have been reported, this study presents a novel activity, suggesting that SAC may reduce the oxidative stress and may thus be useful for pathologies associated with reactive oxygen species.

In conclusion, our data demonstrate that SAC can protect endothelial cells from Ox-LDL-induced injury by preventing the depletion of intracellular GSH and by removing peroxides. These data suggest that SAC may be a useful protective agent against cytotoxicity associated with Ox-LDL.

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