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Antioxidative and Antiglycative Effects of Six Organosulfur Compounds in Low-Density Lipoprotein and Plasma

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Low-density lipoprotein (LDL) and plasma were isolated from patients with non-insulin-dependent diabetes. The protective effects of six organosulfur compounds (DAS, diallyl sulfide; DADS, diallyl disulfide; SAC, *S*-allylcysteine; SEC, *S*-ethylcysteine; SMC, *S*-methylcysteine; SPC, *S*-propylcysteine) against further oxidation and glycation in these already partially oxidized and glycated samples were studied. DAS and DADS showed significantly greater oxidative-delaying effects than four cysteine-containing compounds in both partially oxidized LDL and plasma samples ($P < 0.05$). However, cysteine-containing agents were superior to DAS and DADS in delaying glycative deterioration in already partially glycated LDL ($P < 0.05$). The observed delays of oxidative and glycative effects from each agent were significantly concentration-dependent ($P < 0.05$). Furthermore, six organosulfur agents significantly decreased the loss of catalase and glutathione peroxidase activities in plasma and increased α-tocopherol retention in LDL and plasma ($P < 0.05$). These results suggested that the use of these organosulfur agents derived from garlic at these concentrations could protect partially oxidized and glycated LDL or plasma against further oxidative and glycative deterioration, which might benefit patients with diabetic-related vascular diseases.

KEYWORDS: Glycation; oxidation; organosulfur agents

INTRODUCTION

Hyperglycemic condition can elevate oxidative stress in plasma and low-density lipoprotein (LDL) and increase the production of 8-epi-$PGF_{2\alpha}$ in plasma (*1*, *2*). It is also known that LDL oxidation and glycation are closely interrelated, and both are important factors contributing to the development of diabetic and cardiovascular pathogenesis (*3*−*6*). Because the oxidation and/or glycation occurring in LDL and plasma could further enhance the development of diabetic complications; the use of a proper agent(s) with both antioxidative and antiglycative properties may benefit patients by alleviating oxidative and glycative damages.

Our recent studies have demonstrated that two lipophilic organosulfur compounds, diallyl sulfide (DAS) and diallyl disulfide (DADS), and two hydrophilic organosulfur compounds, *N*-acetylcysteine (NAC) and *S*-ethylcysteine (SEC), on the basis of marked reducing power, metal chelating capability, and ability to act as superoxide ion scavengers, exhibited concentration-dependent antioxidative protection for isolated human erythrocytes and platelets against glucose-induced oxidation (*7*, *8*). Furthermore, another study found that these natural organosulfur agents derived from garlic are able to protect native LDL against oxidation and glycation (*9*). However, it remains unknown if these organosulfur compounds are able to delay or retard the oxidative and glycative deterioration in already partially oxidized and glycated LDL or plasma.

The major purpose of this study was to examine the antideteriorative effects of six organosulfur compounds derived from garlic on partially oxidized LDL, glycated LDL, and oxidized plasma, which were obtained from patients with non-insulin-dependent diabetes mellitus (NIDDM). The influence of these agents on antioxidant enzyme activity and α-tocopherol level in these samples was also evaluated.

MATERIALS AND METHODS

Subjects and Blood Sample Collection. Informed consent for study participation was obtained from 36 patients with non-insulin-dependent diabetes mellitus in Chungshan Medical University Hospital (Taichung City, Taiwan). These patients were not affected by inflammatory or macrovascular diseases. Clinical characteristics of subjects are summarized in **Table 1**. A peripheral blood sample, 15 mL, from each subject was drawn after an overnight fasting. The LDL fraction and plasma of each blood sample were separated. Then, the levels of LDL oxidation, LDL glycation, plasma oxidation, plasma α-tocopherol, and

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Table 1. Clinical Characteristics of the Study Subjects[a]

	NIDDM patients
N	36
sex (men/women)	20/16
age (years)	54.2 ± 8.7
duration (years)	6.5 ± 7.1
BMI (kg/m^2)	24.5 ± 1.9
glucose (mg/dL)	217.8 ± 75
HbA1c (%)	9.5 ± 2.0
cholesterol (mg/dL)	208 ± 52
HDL (mg/dL)	47.3 ± 11.2
LDL (mg/dL)	113 ± 25
triglyceride (mg/dL)	172.7 ± 91
creatinine (mg/dL)	0.96 ± 0.29

[a] Data are means ± SD.

Table 2. Level of Oxidation, Glycation, α-Tocopherol, and Enzyme Activity in 36 Blood Samples before Experiment

LDL oxidation[a] (nmol/mg of LDL protein)	9.5 ± 2.7
LDL glycation (%)	8.4 ± 2.2
plasma oxidation[b] (pg/mL)	27.2 ± 2.9
LDL α-tocopherol content (nmol/mg of LDL protein)	16.4 ± 1.8
plasma α-tocopherol content (μmol/L)	21.7 ± 3.2
plasma catalase activity (units/mg of protein)	6.3 ± 1.2
plasma glutathione peroxidase activity (units/mg of protein)	7.4 ± 1.8

[a] LDL oxidation was determined by MDA content and expressed as nmol/mg of LDL protein. [b] Plasma oxidation was determined by free 8-epi-PGF$_{2\alpha}$ level and expressed as pg/mL.

activity of catalase and glutathione peroxidase in plasma were determined according to the methods described as follows and are presented in **Table 2**.

Organosulfur Compound Analysis and Treatment. DAS (purity = 97%) and DADS (purity = 80%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). DAS was further purified by fractional distillation; the purity was determined according to an HPLC method described in ref *10*, and DAS with purity of 95% was used in this study. *S*-Methylcysteine (SMC, 99%) and *S*-ethylcysteine (SEC, 99.5%) were purchased from Sigma Chemical Co. (St. Louis, MO). *S*-Allylcysteine (SAC) and *S*-propylcysteine (SPC) were supplied by Wakunaga Pharmaceutical Co. (Hiroshima, Japan). Before the experiment, the content of six organosulfur compounds in LDL and plasma was analyzed according to the methods described in refs *10*−*12*. Results showed that no detectable amount of any organosulfur agent was found in these samples. To evaluate the influence of concentration upon the antioxidative and antiglycative effects, 5, 10, and 15 μM of each organosulfur agent were used. On the basis of lipid solubility, DAS or DADS was dissolved in methanol first and then added into plasma or LDL suspension for final concentration preparation. The influence of methanol residue upon lipid oxidation in these suspensions was examined, and it was not significant (data not shown). Other cysteine-containing compounds were directly added into plasma or LDL suspension.

LDL Preparation and Oxidation. LDL fractions with densities of 1.006−1.063 were isolated from plasma by sequential ultracentrifugation (*13*). The isolated LDL was dialyzed against 1.5 mM phosphate-buffered saline (PBS) and sterilized with a 0.22 μM filter. The protein concentration of LDL was determined according to the assay of Lowry et al. (*14*) using bovine serum albumin as a standard. The LDL fraction was diluted to a final concentration of 500 μg of protein/mL using PBS. The method of Jain and Palmer (*15*) was used to measure malondialdehyde (MDA) formation in LDL. Briefly, a 0.2 mL LDL solution was suspended in 0.8 mL of PBS. Then, 0.5 mL of trichloroacetic acid (30%) was added. After vortexing and standing in ice for 2 h, samples were centrifuged at 2000 rpm for 15 min. One milliliter of supernatant was mixed with 0.25 mL of thiobarbituric acid (TBA; 1%), and the mixture was kept in a boiling water bath for 15 min. The concentration of MDA−TBA complex was assayed using an HPLC (Hitachi) equipped with a reverse-phase Shodex KC-812 column with the UV−vis detector at 532. The MDA content in LDL samples from DM patients was 9.5 ± 2.7 nmol/mg of LDL protein (n = 36). Then, these samples were treated with or without organosulfur agents, and further oxidation was initiated with 50 mM glucose at 37 °C; the oxidation level was determined after 72 h of incubation.

LDL Glycation. The method of Duell et al. (*16*) was used to measure the degree of LDL glycation. Briefly, 500 μL of LDL solution was loaded on a Glycogel II column, and glycated LDL was eluted with 2 mL of sorbitol buffer, pH 10.25. Two hundred microliters of eluate (glycated LDL solution) was mixed with 200 μL of 4% $NaHCO_3$ and 200 μL of 0.1% trinitrobenzoic acid. This mixture was flushed with N_2, sealed, and incubated at 37 °C in the dark. After 2 h, the absorbance at 340 nm was measured spectrophotometrically. The blank was a mixture of LDL solution and $NaHCO_3$ in PBS. The LDL glycation level of samples from 36 DM patients was 8.4 ± 2.2%. Further LDL glycation was performed according to the method described in Li et al. (*17*). Glucose at 50 mM was directly added into LDL solutions with and without organosulfur compound treatment. Sodium azide at 0.02% was used as antibiotic to prevent bacterial growth. This solution was sterile filtered, covered with N_2, and stored for 6 days at 37 °C in the dark. After 6 days, the glycative level was determined again. During glycation, samples were treated with ethylenediaminetetraacetic acid (EDTA; 0.5 mM) to prevent the interference from LDL oxidation.

Plasma Oxidation. The plasma level of free 8-epi-PGF$_{2\alpha}$ was measured according to the method of Morrow et al. (*18*). Briefly, each plasma sample was acidified to pH 3 and applied to a C18 Sep-Pak column (Waters, St. Quentin en Yvelines, France). Water, acetone, and heptane were used to wash this column. The collected eluate was then applied to a silica Sep Pak column, in which 8-epi-PGF$_{2\alpha}$ was eluted with ethyl acetate/heptane (50:50, v/v). The final eluate was dried under N_2 and reconstituted in 1 mL of phosphate buffer containing 1% bovine serum albumin. The samples were assayed using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). Ellman's reagent was added to the well after washing, and the absorbance at 405 nm was measured. The plasma 8-epi-PGF$_{2\alpha}$ level from 36 DM patients was 27.2 ± 2.9 pg/mL. These samples were then treated with or without organosulfur agents, and further oxidation was initiated by incubation with 50 mM glucose at 37 °C; the 8-epi-PGF$_{2\alpha}$ level was determined after 72 h of incubation.

α-Tocopherol Analysis. The concentration of α-tocopherol in LDL and plasma was measured according to the HPLC method of Palozza and Krinsky (*19*). The limitation of detection was 3 μmol/L. The α-tocopherol content in LDL samples was 16.4 ± 1.8 nmol/mg of LDL protein. In plasma, the α-tocopherol content was 21.7 ± 3.2 μmol/L.

Catalase and Glutathione Peroxidase (GPX) Assay. The protein concentration of plasma was determined according to the assay of Lowry et al. (*14*) using bovine serum albumin as a standard. Then, plasma was diluted to a final concentration of 1 mg of protein/mL using PBS. Catalase and GPX activities (units per milligram of protein) in plasma were determined by using commercial kits (Calbiochem, EMD Biosciences, Inc., San Diego, CA).

Statistical Analyses. The effect of each treatment was analyzed from 36 different preparations (n = 36). Data were subjected to analysis of variance (ANOVA) and computed using the SAS General Linear Model (GLM) procedure (*20*). Differences among means were determined by using the least significant difference test with significance defined at $P < 0.05$.

RESULTS

As presented in the control groups of **Tables 3** and **4**, the presence of glucose at 50 mM significantly enhanced oxidation and/or glycation development in LDL and plasma ($P < 0.05$). Delayed oxidative deterioration in LDL and plasma by six organosulfur compounds is shown in **Table 3**. Both DAS and DADS showed significantly greater antioxidative protection than four cysteine-containing compounds in these partially oxidized samples against MDA and 8-epi-PGF$_{2\alpha}$ formations ($P < 0.05$).

Table 3. Effects of Six Organosulfur Compounds[a] at 5, 10, and 15 μM against 50 μM Glucose-Induced MDA Formation and 8-epi-$PGF_{2\alpha}$ Formation in Partially Oxidized LDL and Plasma after 72 h of Incubation at 37 °C[b]

	MDA (nmol/mg of LDL protein)			8-epi-$PGF_{2\alpha}$ (pg/mL)		
	5 μM	10 μM	15 μM	5 μM	10 μM	15 μM
control	18.2 ± 2.1c	17.8 ± 2.3c	18.1 ± 2.0d	50.5 ± 4.7d	49.3 ± 4.4c	51.0 ± 4.8d
DAS	13.8 ± 1.7a	11.3 ± 1.7a	10.4 ± 1.1b	42.2 ± 4.1b	37.4 ± 3.3a	33.6 ± 2.7b
DADS	12.7 ± 1.4a	10.7 ± 1.4a	8.8 ± 0.8a	40.6 ± 3.3a	35.6 ± 2.8a	30.7 ± 1.6a
SAC	15.5 ± 0.7b	13.8 ± 1.5b	12.1 ± 1.3c	46.7 ± 2.8c	40.3 ± 4.1b	37.9 ± 2.9c
SEC	16.0 ± 1.2b	14.2 ± 1.8b	12.7 ± 0.9c	45.3 ± 3.1c	42.0 ± 2.6b	39.0 ± 1.4c
SMC	15.7 ± 1.6b	14.5 ± 2.1b	11.5 ± 1.0c	44.8 ± 1.9c	41.7 ± 3.8b	38.4 ± 3.0c
SPC	15.5 ± 1.2b	13.6 ± 2.0b	11.2 ± 0.7c	43.6 ± 2.6b	39.3 ± 2.2b	33.5 ± 2.3b

[a] DAS, diallyl sulfide; DADS, diallyl disulfide; SAC, *S*-allylcysteine; SEC, *S*-ethylcysteine; SMC, *S*-methylcysteine; SPC, *S*-propylcysteine. [b] Controls were the samples without organosulfur compound treatment. Values in the same column with different letters are significantly different ($P < 0.05$).

Table 4. Effects of Six Organosulfur Compounds[a] at 5, 10, and 15 μM against 50 μM Glucose-Induced Glycation in LDL Samples after 6 Days at 37 °C in the Dark[b]

	glycation (%)		
	5 μM	10 μM	15 μM
control	18.5 ± 1.4d	18.3 ± 1.2d	19.1 ± 1.4c
DAS	16.3 ± 0.9c	14.1 ± 1.0c	12.1 ± 1.2b
DADS	14.9 ± 1.1b	13.7 ± 0.5c	11.7 ± 0.8b
SAC	12.6 ± 0.7a	10.2 ± 0.8a	9.2 ± 0.5a
SEC	13.0 ± 1.2a	11.7 ± 1.1b	9.6 ± 0.9a
SMC	14.6 ± 1.0b	12.1 ± 0.7b	10.3 ± 0.7a
SPC	14.5 ± 0.6b	10.6 ± 0.9a	8.9 ± 1.2a

[a] DAS, diallyl sulfide; DADS, diallyl disulfide; SAC, *S*-allylcysteine; SEC, *S*-ethylcysteine; SMC, *S*-methylcysteine; SPC, *S*-propylcysteine. [b] Controls were the LDL samples without organosulfur compound treatment. Values in the same column with different letters are significantly different ($P < 0.05$).

Table 5. Influence of Six Organouslfur Compounds[a] at 10 μM on α-Tocopherol Retention in Already Oxidized LDL and Plasma Samples after 72 h of Incubation at 37 °C[b]

	LDL (nmol/mg of LDL protein)	plasma (μmol/mL)
control	6.2 ± 1.6a	1.1 ± 1.7a
DAS	10.8 ± 1.4b	18.1 ± 2.1d
DADS	9.5 ± 1.0b	19.8 ± 2.0d
SAC	13.8 ± 2.0c	15.6 ± 1.7c
SEC	14.4 ± 1.5c	14.8 ± 1.9b
SMC	12.8 ± 1.7c	13.3 ± 2.0b
SPC	13.8 ± 2.1c	14.0 ± 1.6b

[a] DAS, diallyl sulfide; DADS, diallyl disulfide; SAC, *S*-allylcysteine; SEC, *S*-ethylcysteine; SMC, *S*-methylcysteine; SPC, *S*-propylcysteine. [b] Controls were the LDL samples without organosulfur compound treatment. Values in the same column with different letters are significantly different ($P < 0.05$).

However, the four cysteine-containing agents were superior to DAS and DADS in delaying glycative development in already partially glycated LDL ($P < 0.05$, **Table 4**). The delays of glycative and oxidative effects by each organosulfur agent were concentration-dependent ($P < 0.05$).

The influence of organousulfur agents upon α-tocopherol content in LDL and plasma is presented in **Table 5**. The addition of four cysteine-containing agents significantly increased α-tocopherol retention in LDL when compared with control, DAS, and DADS groups ($P < 0.05$). However, DAS and DADS treatments resulted in greater α-tocopherol retention in plasma than four cysteine-containing agents ($P < 0.05$). The effect of organousulfur agents upon catalase and glutathione peroxidase activities in plasma is shown in **Table 6**. The treatment of 50 mM glucose significantly decreased the activities of catalase and glutathione peroxidase ($P < 0.05$). However, the presence

Table 6. Effects of Six Organosulfur Compounds[a] at 10 μM on Plasma Catalase and Glutathione Peroxidase (GPX) Activities in Already Oxidized Plasma Samples after 72 h of Incubation at 37 °C[b]

	catalase (units/mg of protein)	GPX (units/mg of protein)
control	3.4 ± 1.2a	4.5 ± 1.5a
DAS	5.6 ± 1.4c	6.4 ± 2.0c
DADS	5.8 ± 1.3c	6.8 ± 1.8c
SAC	5.3 ± 1.1c	5.8 ± 1.3b
SEC	4.7 ± 1.6b	5.5 ± 1.5b
SMC	5.0 ± 1.2b	5.3 ± 1.0b
SPC	5.5 ± 1.4c	6.0 ± 1.6c

[a] DAS, diallyl sulfide; DADS, diallyl disulfide; SAC, *S*-allylcysteine; SEC, *S*-ethylcysteine; SMC, *S*-methylcysteine; SPC, *S*-propylcysteine. [b] Controls were the samples without organosulfur compound treatment. Values in the same column with different letters are significantly different ($P < 0.05$).

of these organosulfur agents significantly delayed the activity loss of two antioxidant enzymes ($P < 0.05$), in which the effects of DAS and DADS were greater than those of cysteine-containing agents.

DISCUSSION

LDL oxidation, LDL glycation, and plasma oxidation are the consequence of poor glycemic control in diabetic patients (*1*−*4*), and these consequences could further enhance the development of diabetic complication and other vascular diseases (*21*, *22*). Our past study has found that DAS, DADS, and SEC were able to protect native LDL, erythrocyte membranes, and platelets against oxidation and/or glycation (*7*−*9*). The major finding in the present study was that DAS, DADS, SEC, and another three organosulfur agents derived from garlic were able to delay further oxidative and glycative deterioration in already partially oxidized and/or glycated LDL and plasma. It is believed that the antideteriorative effects from these agents could provide more medical benefit for DM patients to delay or retard the development of diabetic complications or cardiovascular diseases. Further in vivo study is necessary to verify the antioxidative and antiglycative protection of these agents. These organosulfur agents are compounds naturally formed in *Allium* foods such as garlic, Chinese leek, and onion. Thus, the use of these agents at these concentrations as antioxidative and/or antiglycative agents might be safe. However, it has been indicated that the excess dietary consumption of SMC caused growth depression in rats (*23*). Therefore, the concentrations used of these agents should be carefully considered.

The addition of six organosulfur agents to LDL and plasma increased α-tocopherol retention and decreased the activity loss of catalase and GPX. It is believed that the spared α-tocopherol and retained antioxidant enzyme activities consequently con-

tributed to the observed antioxidant protection in LDL and plasma. Our previous studies observed that SEC was superior to DAS and DADS in sparing α-tocopherol in liposomes and native human LDL and suggested that lipophilicity difference between two antioxidant agents was an important role in determining their interaction (*7*, *9*). However, it was interesting to find that four cysteine-containing agents spared greater α-tocopherol in LDL particles, but DAS and DADS spared more α-tocopherol in plasma in our present study. Apparently, α-tocopherol showed an interactive behavior different from that of these organosulfur agents in LDL and plasma. LDL is a lipid-rich environment, and α-tocopherol is strongly associated with lipid components of the LDL particle; however, plasma is a water-rich environment, and α-tocopherol is independently associated with other lipid molecules in plasma. These environmental factors might interfere in the interaction of α-tocopherol with other agents.

The four hydrophilic agents also exhibited greater antiglycative effects than two lipophilic agents in LDLs. These cysteine-containing agents are amino acid-based hydrophilic compounds and might possess greater affinity to water soluble molecules such as glucose; that is, they may compete with the protein part of the LDL particle for glucose, which would allow them to interfere with further glycation development between glucose and the LDL protein part. In the present study, four cysteine analogues were used and showed similar antioxidant and antiglycative protection in isolated human blood. However, our previous study observed that these analogues exhibited different antioxidant activities in mice blood (*24*). Therefore, the relationship between the structure and activity of these agents needs further study.

In the present study, the LDL and plasma used were already partially oxidized or glycated. Apparently, free radicals and glycated products were already present in these samples. Therefore, the observed antideteriorative effects from these organosulfur agents might be due to the fact that these agents protected unoxidized and unglycated samples against the attack from free radicals and glycated products, which retarded further oxidative and glycative development. The other possibility was that these agents scavenged already existing free radicals or glycated products, which decreased the flux of reactive oxygen species and alleviated further oxidative and/or glycated deterioration. Our recent study has indicated that these organosulfur agents possessed nonenzymatic antioxidative activities such as reducing power, metal-chelating capability, and superoxide ion scavenging effect (*7*−*9*). Thus, it was highly possible that these agents protected these already partially oxidized samples against further oxidative damage via these actions.

In conclusion, six organosulfur compounds derived from garlic showed marked antioxidative and antiglycative effects in partially oxidized (or glycated) LDL and plasma against further deterioration. The use of these agents could delay oxidation and glycation development and reduce related damage, which may benefit patients with vascular diseases.

ABBREVIATIONS USED

DAS, diallyl sulfide; DADS, diallyl disulfide; SAC, *S*-allylcysteine; SEC, *S*-ethylcysteine; SMC, *S*-methylcysteine; SPC, *S*-propylcysteine; NIDDM, non-insulin-dependent diabetes mellitus; NAC, *N*-acetylcysteine; MDA, malondialdehyde; GPX, glutathione peroxidase; LDL, low-density lipoprotein; EDTA, ethylenediaminetetraacetic acid.

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