Antioxidative Effect of S-allylmercaptocysteine Derived from Aged Garlic on Oxidation of Human Low Density Lipoprotein

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Oxidation of low density lipoprotein (LDL) plays an important role in the development and progression of atherosclerotic disease. In this study, human LDL was isolated and oxidized using CuSO4 in the presence or absence of S-allylmercaptocysteine. Oxidative modification of the LDL fraction was monitored by both the appearance of thiobarbituric acid substances (TBARS), an increase in electrophoretic mobility, and conjugated diene formation. The addition of S-allylmercaptocysteine reduced lipid peroxide formation, indicating it to be an effective antioxidant. The inhibition of LDL oxidation by 5~20 μg/ml S-allylmercaptocysteine occurred in a dose-dependent manner, as assessed by the TBARS assay. S-allylmercaptocysteine at 20 μg/ml almost completely inhibited the Cu²⁺ induced increases in electrophoretic mobility of LDL and almost completely inhibited conjugated diene formation. A more potent antioxidative activity was observed for S-allylmercaptocysteine than for either Vitamin C or dl-α-tocopherol. Thus, S-allylmercaptocysteine aid in preventing the development and progression of atherosclerotic disease.

Key words: Low density lipoprotein, aged garlic, S-allylmercaptocysteine, antioxidative activity

Introduction

The oxidation of low density lipoprotein (LDL) is believed to be critical process in the development of atherosclerosis [2,19]. Oxidized LDL (Oxi-LDL) exerts a myriad of effects including monocyte chemotaxis, decreased macrophage mobility, and ultimately, foam cell formation [1]. In turn, the recruitment and subsequent activation of these circulating macrophages results in the production of free radical and inflammatory cytokines [6]. Once an atheroma has developed, Oxi-LDL can produce surface fissures, resulting in larger and more exclusive atherosclerosis [27,28].

Inevitably, atherogenic progression originates upon an oxidation of the LDL particle, resulting in the transformation into a pathophysiological entity, Oxi-LDL [7,20]. Antioxidants have been the focus of recent studies involving the atherogenic process, primarily because of the oxidant-dependent nature of atherogenic initiation. In recent years, garlic and garlic compounds have received increased attention because of the possible beneficial effects in reducing the LDL oxidation [12,18,21]. Strong evidence has suggested that many of these beneficial properties may be attributed to specific compounds found in garlic and its extracts. Studies involving chemical analysis of garlic suggest that organosulfur compounds are responsible for its bioactivity [11,14,21]. These compounds pose a reducing sulfur center attached to organic side groups, enabling stabilization of their negative charge. This combination in chemical structure thus confers strong antioxidant properties upon some of these compounds. S-allylmercaptocysteine is one of the major metabolite compound in aged garlic [14,15].

S-allylmercaptocysteine is biotransformed from allyl sulfides and from the naturally occurring water soluble sulfur compound during aged garlic preparation (Fig. 1) [23].

S-allylmercaptocysteine has been demonstrated to have antioxidant properties [17,26], prevention of liver damage [29,30], and prostate cancer [3,10,32]. Synthetic antioxidants, such as the drug, probucol have been prescribed as an adjunct therapy along with other vasodilator [13]. However, their toxicity limits their usage and ultimately, their potential as therapeutic agents. On the other hand, many naturally occurring antioxidants derived from edible plant products offer similar protection, without the associated toxicity.
Fig. 1. Formation of S-allylmercaptocysteine (SAMC) from garlic-derived polysulfides [23].

Following these considerations, this paper focuses antioxidative activity for Oxi-LDL by S-allylmercaptocysteine derived from aged garlic, which might have an important role in the benefits of garlic to human health.

Materials and Methods

Chemicals
S-allylmercaptocysteine (S-allylmercaptocysteine 85% pure, plus 5% S-propenylmercaptocysteine, and 10% S-methylmercaptocysteine) was provided by Sejong University, other chemicals were purchased from Sigma Co. (St. Louis, USA).

Isolation of human low density lipoprotein (LDL)

Human LDL was isolated from blood of healthy man by ultracentrifugation and dialyzed extensively against 0.9% (w/v) NaCl, 0.004% (w/v) EDTA, pH 7.4 [9]. Prior to oxidation, LDL was dialyzed against phosphate-buffered saline, pH 7.4 to remove the EDTA.

Oxidation of LDL

Oxidation was made by exposing LDL (100 μg/ml) to 5 μM CuSO₄ in 2 mM phosphate buffer pH 7.5, at 37°C for incubation. For antioxidative effect, these incubation and relevant control were performed in the presence of 5, 10, 15 and 20 in g/ml of S-allylmercaptocysteine. At time intervals, aliquots of the reaction mixture were taken to measure the extent of oxidation evaluating the thiobarbituric acid reactive substances (TBARS) [24].

Assay of thiobarbituric acid reactive substances (TBARS)

TBARS levels were determined spectrophotometrically. To 0.1 ml aliquots of post incubation mixture and also tetramethoxypropane standards were added 1 ml of 20% trichloroacetic acid and 1 ml of 1% thiobarbituric acid containing EDTA. Tubes were placed in a boiling water bath for 30 min. After cooling, tubes were centrifuged at 1,500 × g for 15 min. Absorbance of the supernatant was measured at 532 nm [33].

LDL gel electrophoresis

Electrophoresis of oxidized and native LDL was carried out on agarose gel in barbital buffer, pH 8.6. The agarose plates were then stained with Nile red [8]. Results are expressed as relative electrophoretic mobilities compared with the migration of native LDL.

Detection of conjugated dienes

The formation of conjugated dienes associated with oxidized LDL was measured by monitoring at 234 nm using a UV-VIS spectrophotometer [4]. Briefly, 1 ml LDL solution (100 μg LDL protein/ml) in phosphate buffer saline, pH 7.4 was incubated with 5 μM CuSO₄ at 37°C in both the presence or absence of samples, and the absorbance at 234 nm was measured every 30 min. The formation of conjugated dienes in control solution containing antioxidant in the absence of 5 μM CuSO₄ was also measured.

Determination of protein

The protein was determined by the methods of Lowry, et al. [16].

Statistics

Data in tables and figures are mean±SD. Statistical significance was examined through one way analysis of variance. Significance differences were accepted at p<0.05.

Results and Discussion

Antioxidative effects on human LDL oxidation

Transition metal including copper and iron have been shown to be strong catalysts for LDL oxidation in vitro. Cu²⁺ ion was found to be effective at initiating the oxidation of EDTA-free human LDL as measured by TBARS. Although the physiological significance of in vitro Cu²⁺ induced LDL
oxidation remains controversial, this method has been a useful model for evaluating naturally occurring antioxidant compound [5].

Antioxidative effects of S-allylmercaptocysteine on the oxidation of LDL, as measured by production of TBARS. To obtain the Oxi-LDL, LDL was oxidized with Cu2+ in the time dependent manner and the production of TBARS reached a plateau after 5 hr of incubation. Upon coinubation with various concentration of S-allylmercaptocysteine, its effects on TBARS formation was most noticeable at 10 and 20 μg/ml (Fig. 2). LDL lipid peroxidation in the presence of Cu2+ was increased approximately 6 fold in comparison to its relevant control. The presence of 10 μg/ml or 20 μg/ml of S-allylmercaptocysteine significantly inhibited lipid peroxidation by approximately 58% and 79%, respectively.

S-allylmercaptocysteine possesses a reducing sulfur center attached to organic side groups, enabling stabilization of their negative charge. This combination in chemical structure thus confers strong antioxidant properties upon some of those compounds [14]. S-allylmercaptocysteine is recognized as a significant allyl sulfur compound occurring in aged garlic preparation [14,15].

Although there is little evidence for the actual involvement of Cu2+ in modifying in vivo atherosclerotic lesions have been shown to contain detectable amounts of redox reactive copper [25]. This catalytic copper may contribute to the prooxidant environment of the atherosclerotic lesion where extensive LDL oxidation take place. The ability of S-allylmercaptocysteine to complex copper may reduce in vivo. It has also been reported that LDL isolated from subjects who have consumed aged garlic or other plant extracts is less susceptible to in vivo oxidation [25]. There observations suggest that S-allylmercaptocysteine present in aged garlic remain active after ingestion and enter the circulation where it may exert an antioxidant effect of decreasing the lipid content of LDL [31] and/or interacting with endogenous antioxidants [22].

Effects of S-allylmercaptocysteine on LDL oxidation by electrophoretic mobility

LDL oxidized by Cu2+ displayed a greater electrophoretic mobility in agarose gel compared to native LDL. Oxidation of the LDL by Cu2+ greatly increased its electrophoretic mobility (Table 1). This increase in electrophoretic mobility was completely abolished in the presence of 20 μg/ml S-allylmercaptocysteine. However, this striking effect was effect observed at concentration of 5-10 μg/ml S-allylmercaptocysteine. S-allylmercaptocysteine did induce a concentration dependent decrease in the electrophoretic mobility of LDL, but, even at concentrations of 5 μg/ml S-allylmercaptocysteine slightly inhibited the Cu2+ induced increase in LDL electrophoretic mobility. Steinbrecher et al. [28] demonstrated that LDL can be modified by the addition

![Fig. 2. Effects of S-allylmercaptocysteine (SAMC) on Cu2+-induced LDL oxidation. LDL (100 μg protein/ml) was incubated for 5 hr at 37°C with phosphate buffer containing 5 μM was CuSO4 in the absence (□) or presence of SAMC (▲). Significant differences (p<0.05) from only are indicated by *. Significant differences (p<0.05) from LDL+Cu2+ are indicated by **.](image)

Table 1. Inhibition effect of S-allylmercaptocysteine on oxidation assessed by electrophoretic mobility

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Relative electrophoretic mobility</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native LDL</td>
<td>1.0±0.02</td>
<td></td>
</tr>
<tr>
<td>Native LDL + Cu2+</td>
<td>4.02±0.03</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Native LDL + Cu2+ + SAMC 1 μg/ml</td>
<td>2.60±0.03</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Native LDL + Cu2+ + SAMC 5 μg/ml</td>
<td>2.03±0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Native LDL + Cu2+ + SAMC 10 μg/ml</td>
<td>1.85±0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Native LDL + Cu2+ + SAMC 20 μg/ml</td>
<td>1.24±0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

aLDL (100 μg/ml) was incubated for 5 hr with 5 μM CuSO4 in the absence or presence of S-allylmercaptocysteine (SAMC). The electrophoretic mobility of LDL was determined in agarose gel as described in the text.

bMean±SD (n=6). Value means in a column sharing the same superscript letter are significantly different (p<0.05).
of fatty acid peroxidation in the absence of cells. This modified LDL possesses an enhanced electrophoretic mobility without the lipid constituents of LDL being oxidized. It is possible that oxidation of LDL mediated by Cu²⁺ can also contribute to the modification of the LDL protein as determined by the enhanced electrophoretic mobility.

Effects of S-allylmercaptocysteine on conjugated diene formation

Fig. 3 shows the effect of S-allylmercaptocysteine on the formation of conjugated dienes, a measurement of LDL oxidative process. The conjugated dienes formed was significantly lower in the presence of S-allylmercaptocysteine compared to the control.

S-allylmercaptocysteine at the concentration of 5 μg/ml was less effective in producing a antioxidative activity during early initiation stage after 30–150 min incubation. The antioxidative activity of S-allylmercaptocysteine at 20 μg/ml was slightly stronger than that a 10 μg/ml S-allylmercaptocysteine reduced the lag phase of human LDL oxidation at 20 μg/ml compared to the control, thus indicating that it can contribute to a prooxidant effect in the presence of Cu²⁺.

![Graph showing antioxidative effects of S-allylmercaptocysteine on conjugated diene formation](image)

Fig. 3. Antioxidative effects of S-allylmercaptocysteine on the formation conjugated diene observed during the oxidation of LDL. LDL (100 mg protein/ml) was incubated in the presence or absence of various concentration of S-allylmercaptocysteine. Oxidation was initiated by addition of 5 μM CuSO₄. The formation of conjugated dienes was measured at 234 nm on LDL oxidation. Control (LDL+5 μM CuSO₄), S-allylmercaptocysteine at 5 μg/ml, LDL+5 μM CuSO₄+5 μg/ml, LDL+5 μM CuSO₄+10 μg/ml, LDL+5 μM CuSO₄+20 μg/ml. Mean±SD (n=6), Value means in a column not sharing the same common superscript letters are not significantly different (p<0.05).

Incubation of LDL with Cu²⁺ produced a lag phase of 150 min before the onset of the propagation phase where polyunsaturated fatty acid underwent conversion to conjugated lipid hydroperoxide. However, in the presence of S-allylmercaptocysteine at the concentration of 10 or 20 μg/ml, the lag phase and propagation phase were inhibited. In agreement with the finding Munday, et al. [9], there was an initial lag phase in the formation of conjugated dienes. When higher amounts of S-allylmercaptocysteine were employed, the conjugated dienes not formed than that of control. The Cu²⁺ concentration was coupled with decrease of conjugated diene formation, which is an index of a lipid propagation phase and dependently only on the lipid composition of LDL. The presence of 10 or 20 μg/ml S-allylmercaptocysteine delayed the reaching of high absorbance and this delays was higher at concentration of 20 μg/ml.

Allicin is rapidly metabolised in the body, first to diallyldisulfide and then to allyl mercaptan, neither of which possesses antioxidative activity [14,29]. Aged garlic, however, contains no allicin [15] and the in vitro antioxidant action of this supplement is believed to be derived largely from its content of S-allylmercaptocysteine [23].

Comparative antioxidative effect of S-allylmercaptocysteine and dl-α-tocopherol

Human LDL was oxidized with Cu²⁺ at the same concentrations of S-allylmercaptocysteine, and dl-α-tocopherol, the production of TBARS reached a plateau after 24 hr of incubation (Fig. 4). Therefore, all data relating to Cu²⁺ presented were obtained following 5 hr incubation. The oxidation of LDL by Cu²⁺ was completely inhibited in the presence of 20 μg/ml S-allylmercaptocysteine. The potency of S-allylmercaptocysteine, dl-α-tocopherol were of the same magnitude.

Moreover, in our system, S-allylmercaptocysteine exhibited a significantly higher effectiveness than Oxi-LDL antioxidant than dl-α-tocopherol. Although the mechanism of oxidation is not known, LDL oxidation involve cellular lipoygenase [31]. The inhibition of Cu²⁺-induced oxidation by antioxidants was consistent with the role of lipoxygenase. Endo cytost and degrade oxidatively modified LDL via scavenger receptor at much greater than native LDL [11]. This property was used to assess the protection afforded LDL through coinuculation with antioxidant during the oxidation period. Protection by antioxidant against cell induced LDL oxidation maybe, in part, due to its capacity to scavenge free
radicals. However, inhibition of Cu± induced LDL oxidation by antioxidant was less marked then the oxidation by macrophage. The difference in antioxidative potency in two systems might due to the fact that oxidative modification is more complex than a simple free radical reaction [25]. To support forth the physiological relevance of these results, S-allylmercaptocysteine derived from aged garlic was compared to Vitamin C and, which can be considerate reference antioxidant in biological system. From these reports, it appear that S-allylmercaptocysteine is important in preventing the oxidative modification of LDL. S-allylmercaptocysteine may also play an important role in preventing the peroxidation of LDL in vivo perhaps through regeneration soluble antioxidant, such as S-allylmercaptocysteine may be important in preventing or reducing the progression of atherosclerosis by inhibition the peroxidation of lipoprotein.

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References

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