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**ARTICLE** *in* PATHOBIOLOGY · FEBRUARY 2005

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Pathobiology 2005;72:325–334
DOI: 10.1159/000091330

Received: April 18, 2005
Accepted: November 22, 2005

# The Antiatherogenic Effect of Allicin: Possible Mode of Action

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**Key Words**

Atherosclerosis · Allicin · Low-density lipoprotein receptor knockout mice · Apolipoprotein E-deficient mice · Lipoproteins

**Abstract**

***Objective:*** Garlic *(Allium sativum)* has been suggested to affect several cardiovascular risk factors. Its antiatherosclerotic properties are mainly attributed to allicin that is produced upon crushing of the garlic clove. Most previous studies used various garlic preparations in which allicin levels were not well defined. In the present study, we evaluated the effects of pure allicin on atherogenesis in experimental mouse models. ***Methods and Results:*** Daily dietary supplement of allicin, 9 mg/kg body weight, reduced the atherosclerotic plaque area by 68.9 and 56.8% in apolipoprotein E-deficient and low-density lipoprotein (LDL) receptor knockout mice, respectively, as compared with control mice. LDL isolated from allicin-treated groups was more resistant to $CuSO_4$-induced oxidation ex vivo than LDL isolated from control mice. Incubation of mouse plasma with $^3H$-labeled allicin showed binding of allicin to lipoproteins. By using electron spin resonance, we demonstrated reduced $Cu^{2+}$ binding to LDL following allicin treatment. LDL treatment with allicin significantly inhibited both native LDL and oxidized LDL degradation by isolated mouse macrophages. ***Conclusions:*** By using a pure allicin preparation, we were able to show that allicin may affect atherosclerosis not only by acting as an antioxidant, but also by other mechanisms, such as lipoprotein modification and inhibition of LDL uptake and degradation by macrophages.



This work was performed in partial fulfillment of the requirements for a PhD degree of A.G., Sackler Faculty of Medicine, Tel-Aviv University, Israel.

## Introduction

Atherosclerosis is the leading cause of morbidity and mortality in Western society.

Atherosclerosis is characterized by accumulation of cholesterol deposits in macrophages in the arteries. This deposition leads to a proliferation of certain cell types within the arterial wall that gradually impinge on the vessel lumen and impede blood flow [1–3]. A critical event in the initiation of atherosclerosis is the accumulation and modification of low density lipoprotein (LDL) in the

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subendothelial matrix [1–6]. An LDL particle is a highly compartmentalized particle, consisting of a monolayer of phospholipids and free cholesterol, a cholesteryl ester and triglyceride core, and lipophilic antioxidants (e.g., vitamin E, β-carotene, and ubiquinol) distributed throughout the particle [1–3, 6]. LDL also contains a single molecule of apolipoprotein B-100 (apoB-100). This protein is made up of 4,536 amino acids, 25 of which are cysteinyl residues. Nine of them exist as free thiols, located in the hydrophobic regions of LDL, and the remainder form disulfide bonds [7].

The LDL particles are in a dynamic state, their structure and physical properties being dependent on their lipid composition as well as on the conformation of apoB-100. It has subsequently been shown that trapped LDLs undergo modifications, including oxidation, lipolysis, proteolysis and aggregation, and that such modifications contribute to inflammation as well as to foam cell formation [1–3].

The mechanism of in vivo oxidation of LDL is still largely unknown. Both metal-dependent and metal-independent mechanisms have been suggested to be involved in this process [3–6, 8–11]. Oxidation of LDL may involve most of its constituent molecules, including cholesterol, fatty acids, phospholipids, antioxidants and apoB [3, 8–11].

The involvement of garlic *(Allium sativum)* constituents in atherogenesis has been controversial. Garlic has been suggested in numerous studies to have positive effects on cardiovascular risk factors [12–25]; however, other studies found no effects [20–22, 26, 27].

The antiatherogenic potential of garlic has been demonstrated in animal models, in both rabbit and mouse atherosclerosis models, in which garlic preparations reduced the manifestation of the disease [15, 17, 19, 21–23].

The antiatherosclerotic-related properties of garlic are attributed mainly to its organosulfur compounds, particularly allicin [20–22, 26, 28]. Allicin produced upon crushing of the garlic clove by the interaction of alliin, the non-protein amino acid, with the pyridoxal phosphate-containing enzyme alliinase as a result of decompartmentalization, brings the enzyme and the substrate into contact in the cytoplasm [20, 22, 29]. A variety of biological effects is attributed to both the sulfhydryl (SH)-modifying and the antioxidant activity of allicin [30, 31].

In a previous study, we showed that diet-induced atherosclerotic lesion, in C57BL/6 mice, was reduced by a daily oral intake of pure allicin as compared with a control group, despite the fact that their plasma lipid profile was not affected at all [24]. On the other hand, Espirito et al. [26, 27] found no effect of allicin on lipid or atherogenesis in APOE*3-Leiden mice.

In the present study, we evaluated the effects of pure allicin on atherogenesis in an experimental model of apolipoprotein E-deficient (apoE–/–) and LDL receptor knockout (LDL-R–/–) mice, and tried to elucidate the possible mechanisms of allicin intervention with atherogenesis.

## Materials and Methods

*Preparation of Allicin*

Allicin (12 mmol/l in 50 mmol/l phosphate buffer, pH 6.5) was prepared as previously described [31]. $^{3}$H-labeled allicin was prepared by first synthesizing $^{3}$H-labeled alliin, followed by enzymatic conversion to allicin and high-performance liquid chromatography purification [32].

*In vitro Studies*

*The Effect of Allicin on LDL.* Human LDL (d = 1.019–1.063 g/ml) was isolated from the blood of healthy volunteers by sequential density ultracentrifugation at 4°C in potassium bromide solutions as previously described [33]. The protein concentration was measured by the Lowry assay [34]. LDL was incubated for 15 min in phosphate-buffered saline (PBS), with or without allicin or alliin (1 mmol/l) or N-ethylmaleimide (12 mmol/l) in the dark at room temperature. The excess was washed by dialysis.

*Lipoprotein Oxidation.* LDL from healthy donors (50 μg/ml) was oxidized at 37°C in the dark by two radical generators, $CuSO_4$ and 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH). LDL oxidation was determined by monitoring the changes in absorbance ($A_{234}$) upon conjugated diene formation [35]. All experiments were repeated at least three times.

*Electron Spin Resonance Measurements.* LDL was incubated for 15 min at room temperature in the dark, with or without 1 mmol/l allicin or 0.1 mmol/l methanethiosulfonate-based spin label (MTS-SL) (1-Oxyl-2,2,5,5,-tetramethyl-$\Delta^3$-pyrroline-3-methyl; Toronto Research Chemicals) freshly dissolved in PBS. Buffer exchange (for removal of excess allicin and MTS-SL) was then carried out by dialyzing at 4°C against 0.1 *M* Tris-buffer without EDTA (pH 7.8). Binding of $Cu^{2+}$ to native LDL (nLDL) or allicin/MTS-SL-pretreated LDL was performed at 2 mg/ml LDL and 0.1 mmol/l $Cu^{2+}$ in a total volume of 200 μl. Measurements were done in a flat spectrometer cell (spectrometer model: Bruker ER-200 D-SRC). The experimental setting was the following: central field 3,500 Gs, sweep width 1,000 Gs, receiver gain $2 \times 10^5$, microwave power 20 mW, modulation amplitude 4 Gs.

*Degradation of LDL by Isolated Macrophages.* Degradation of $^{125}$I-nLDL and $^{125}$I-oxidized LDL (oxLDL) by macrophages was studied in vitro. Human nLDL was radio-iodinated by the method of Salacinski et al. [36]. Oxidation of LDL was performed by incubation of $^{125}$I-nLDL (1 mg of protein/ml in EDTA-free PBS) with $CuSO_4$ (10 μmol/l) for 24 h at 37°C. We examined three allicin treatments: (1) 50 μmol/l allicin was added to the isolated macrophages (cell pretreatment) or (2) to isolated LDL (LDL pretreatment) for 15 min at room temperature in the dark, and the excess

was washed off twice by dialysis. (3) Allicin or alliin (50 μmol/l) was added during the incubation of LDL with the macrophages (in assay).

Mouse peritoneal macrophages were isolated as previously described [37]. Degradation was determined by the method of Goldstein et al. [38]. Briefly, mouse peritoneal macrophages were incubated with indicated concentrations of labeled lipoproteins, followed by determination of the non-iodide trichloroacetic acid-soluble radioactivity. Cell and LDL protein concentrations were determined according to Lowry et al. [34].

*In vivo Studies*

*Mice, Diet and Supplementation.* LDL-R–/– (Jackson Laboratories) and apoE–/– mice (C57BL/6 background; Jackson) were bred in the local animal house (Sheba Medical Center, Tel Hashomer, Israel). All procedures using animals were in accordance with the Sheba Medical Center guidelines.

All mice were administered a daily oral dose of 200 μl PBS, pH 7.4 (control group), or allicin in PBS (3 or 9 mg allicin/kg body weight) (treatment groups). The apoE–/– mice, comprising 8-week-old female mice, were randomly divided into three groups of 12 animals each. All mice were fed a regular chow diet containing 4.5% fat by weight (0.02% cholesterol) (TD19519; Koffolk). The experiment lasted 8 weeks. The LDL-R–/– mice, comprising 12-week-old male or female mice, were used in two sets of experiments. In the first set, we studied the effect of allicin on atherogenesis (n = 39, divided into three treatment groups). In the second experiment, we studied the effect of allicin on the lipoprotein profile (n = 24, divided into two treatment groups). These experiments involved two stages. Stage 1 lasted 1 week, during which the mice were fed a regular chow diet. Stage 2 lasted 6 weeks, during which the mice were fed an atherogenic, Western-type diet containing 15.75% fat (43% saturated fat) and 1.25% cholesterol (TD88137; Harlan). To minimize oxidation, the diets were stored in the dark at 4°C. The mice were fed daily ad libitum.

*Cholesterol Level Determination.* Blood samples were obtained from mice via retroorbital bleeding under anesthesia. Total plasma cholesterol and triacylglycerol levels were determined after a 16-hour fast, using an automated enzymatic technique (Boehringer Mannheim), on day 0 and at the end of the study.

*Fast Protein Liquid Chromatography Analysis of Lipoproteins.* Mouse serum lipoproteins were separated by size exclusion chromatography using a Superose-6 column (1 × 30 cm) on a fast protein liquid chromatography system (Pharmacia), as described previously [39]. Briefly, a 200-μl aliquot of mouse serum was injected onto the column and separated with buffer containing 0.15 mol/l NaCl, 0.01 mol/l $Na_2HPO_4$, and 0.1 mmol/l EDTA, pH 7.5, at a flow rate of 0.5 ml/min. Fifty fractions of 0.5 ml each were collected, with the lipoproteins being contained within tubes 17–40. Fractions 17–20 signify very-low-density lipoprotein (VLDL); fractions 21–30 LDL; fractions 31–40 high-density lipoprotein (HDL).

*Assessment of Atherosclerosis in the Aortic Sinus.* Quantification of atherosclerotic fatty streak lesions was performed by morphometric calculation of the lesion size in the aortic sinus, as previously described [24]. Briefly, at the end of the study, the mice were deprived of food overnight and killed under isoflurane anesthesia. The heart and the upper section of the aorta were removed and the peripheral fat carefully cleaned.

The upper section was embedded in OCT compound (Miles Inc.) and frozen. Every other section (10 μm thickness) throughout the aortic sinus (400 μm) was taken for analysis. The three valve cusps, which are the junctions of the aorta to the heart, identified the distal portion of the aortic sinus. Sections were evaluated for fatty streak lesions after staining with oil red O. Lesion areas per section were counted, using Imagepro software (Media Cybernetics), by an examiner unfamiliar with the tested specimen.

*Mouse Lipoprotein Oxidation.* Lipoproteins (density (d) = 1.063 g/ml, top fraction) were isolated from the pooled plasma of each group. Lipoproteins were oxidized by 15 μmol/l $CuSO_4$ as described above.

*Binding of $^3$H-Allicin to Lipoproteins.* The plasma of LDL-R–/– mice was incubated with $^3$H-allicin ($12 \times 10^6$ dpm) for 15 min at room temperature. Lipoproteins were separated on a Superose-6 column as described above. For each fraction, radioactivity was determined using a β-counter (1600 TR, Packard).

*Statistical Analysis*

Data are shown as mean values ± standard error. Analyses of statistically significant differences between the groups were performed by employing Student's t test, ANOVA, or the Mann-Whitney rank test. $p < 0.05$ was considered statistically significant. SigmaPlot 2000 software was used for all statistical analyses.

## Results

The effect of daily supplementation of pure allicin on atherogenesis was investigated in two atherogenic mouse models, apoE–/– and LDL-R–/– mice.

*Effect of Allicin on the Atherosclerotic Lesion Area*

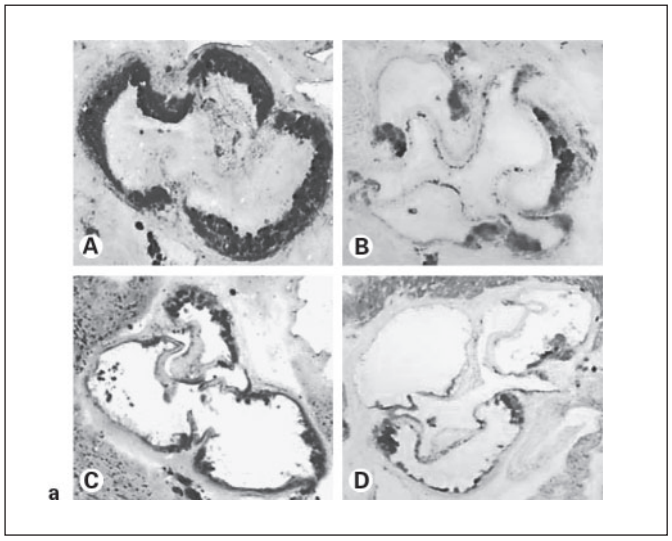
The lesion area at the aortic sinus of mice fed daily with a pure allicin solution was significantly reduced as compared with a control group. Daily supplementation of allicin, 9 mg/kg body weight, reduced the atherosclerotic plaque area by 68.9 and 56.8% in apoE–/– and LDL-R–/– mice, respectively, compared with control mice (fig. 1).

*Body Weight and Plasma Lipid Levels*

No significant difference in body weight was found between the control group and the allicin-fed mice ($p = 0.144$). Analysis of total plasma cholesterol and triglyceride levels at time zero (baseline) and at the end of the study did not show any significant differences between the control group and the allicin-fed mice (data not shown).

*Plasma Lipoprotein Profile*

Following 2 weeks on Western diet, the cholesterol (fig. 2) and triglyceride (data not shown) content of VLDL and LDL in allicin-treated LDL-R–/– mice markedly de-



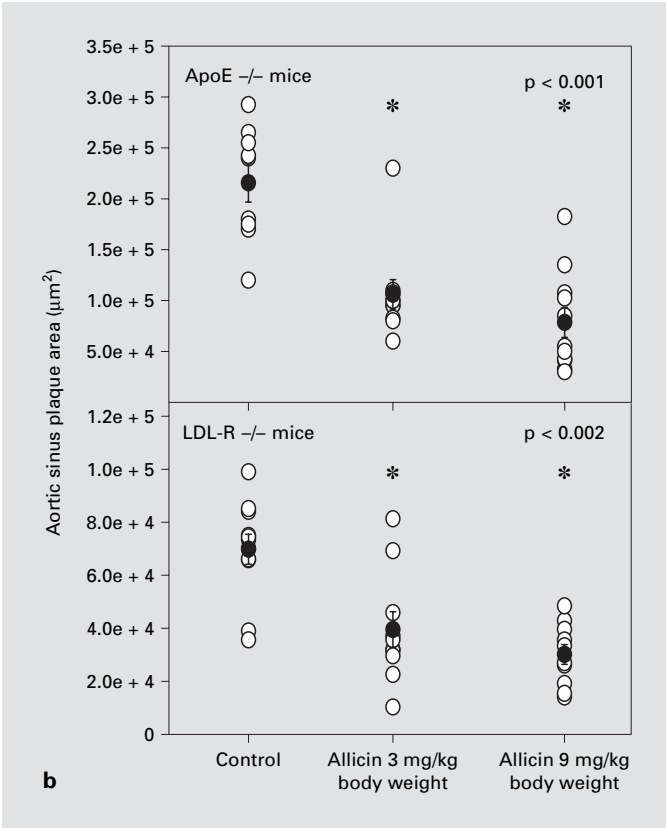




**Fig. 1.** Daily treatment with allicin reduced the atherosclerotic lesion area in apoE–/– and LDL-R–/– mice. **a** Representative photographs of oil red O stained sections from apoE–/– (A, B) and LDL-R–/– mice (C, D). ×40. **b** Quantitative analysis of aortic lesions stained with oil red O and assessed by computerized morphometric measurements. Data represent means ± SEM.

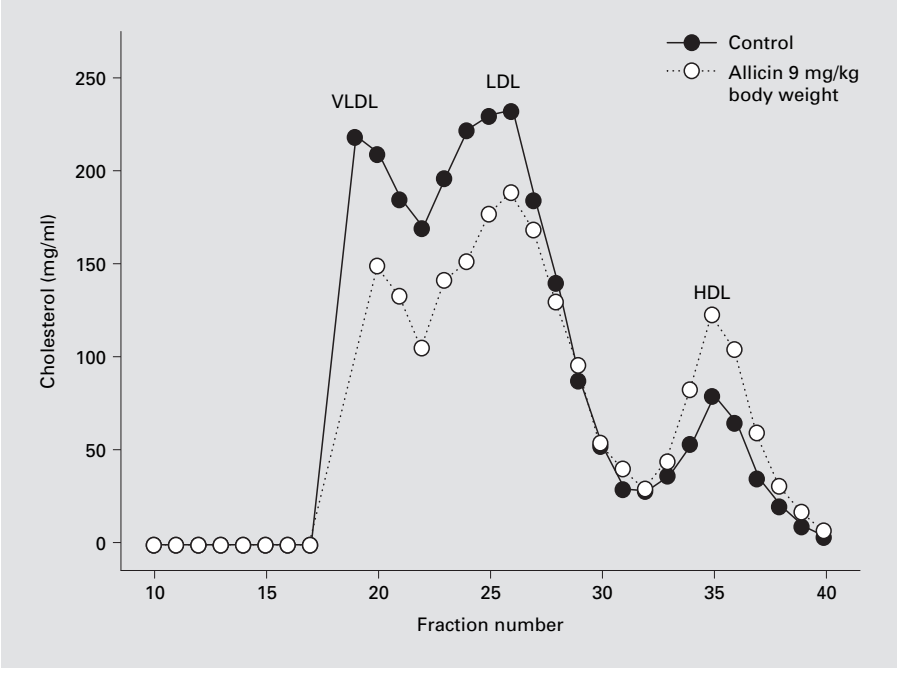



**Fig. 2.** Allicin changes the cholesterol content in lipoproteins. LDL-R–/– mice were fed a regular chow diet with or without allicin (9 mg/kg body weight) followed by atherogenic diet supplemented with a daily dose of allicin or PBS (control). After 2 weeks, plasma was analyzed by size exclusion chromatography. A 200-μl aliquot of mouse plasma was injected into the column and separated as described in 'Materials and Methods'. Fractions of 0.5 ml were collected. Data represent mean values of two blood pools of each treatment group.

creased, while HDL cholesterol increased, as compared with the control group. The calculation of the area under the curve shows that the HDL cholesterol contribution to the total cholesterol is 25.2% in the allicin-treated mice compared with 14.6% in the control mice.

However, after 6 weeks on atherogenic diet, no differences were detected (data not shown).

*Binding of $^3$H-Allicin to Lipoproteins*

Incubation of LDL-R–/– mouse plasma with $^3$H-labeled allicin showed binding of allicin to VLDL, LDL and HDL as demonstrated by size exclusion chromatography (fig. 3).

*Susceptibility of LDL to Oxidation ex vivo*

LDL isolated from the allicin-treated groups, in both apoE–/– and LDL-R–/– mice, was more resistant to $CuSO_4$-induced oxidation ex vivo than LDL isolated from control mice (fig. 4a).

*The in vitro Effect of Allicin on LDL Oxidation*

Lipoprotein oxidation was determined by using two radical generators, $Cu^{2+}$ and AAPH. The results show that allicin, at concentrations higher than 30 μmol/l, effectively inhibits $Cu^{2+}$-induced oxidation of LDL, while at the same concentration, alliin only slightly prolongs the lag phase of LDL oxidation (fig. 4b). AAPH is a water-soluble azo compound that undergoes thermal decomposition and produces peroxyl radicals [11]. AAPH (0.5 mmol/l) induced LDL oxidation at the same rate as $Cu^{2+}$ at 15 μmol/l. In contrast to its strong inhibitory effect on $CuSO_4$-induced LDL oxidation, allicin did not affect AAPH-induced oxidation (fig. 4c). Thus, the inhibition of LDL oxidation by allicin is not a general phenomenon; rather, it depends on the target and degree of oxidation. N-ethylmaleimide, a well-known high-affinity SH ligand, inhibited copper-induced LDL oxidation similarly to allicin (data not shown).

In order to determine the mechanism by which allicin inhibits $Cu^{2+}$-induced LDL oxidation, we examined whether it interferes with the LDL-$Cu^{2+}$ complex formation. Oxidation of LDL by $Cu^{2+}$ is dependent on its binding to LDL [7]. Application of electron spin resonance (ESR) is based on a decreased $Cu^{2+}$ ESR signal as a result of its binding and immobilization on a polymer surface [40]. (The peak intensity of an ESR signal depends on a square of linewidth and is therefore very sensitive to the signal broadening.) We found that in the presence of LDL, the peak intensity of the ESR signal of $Cu^{2+}$ aqua ions decreased by approximately 30 ± 10% (fig. 5). This

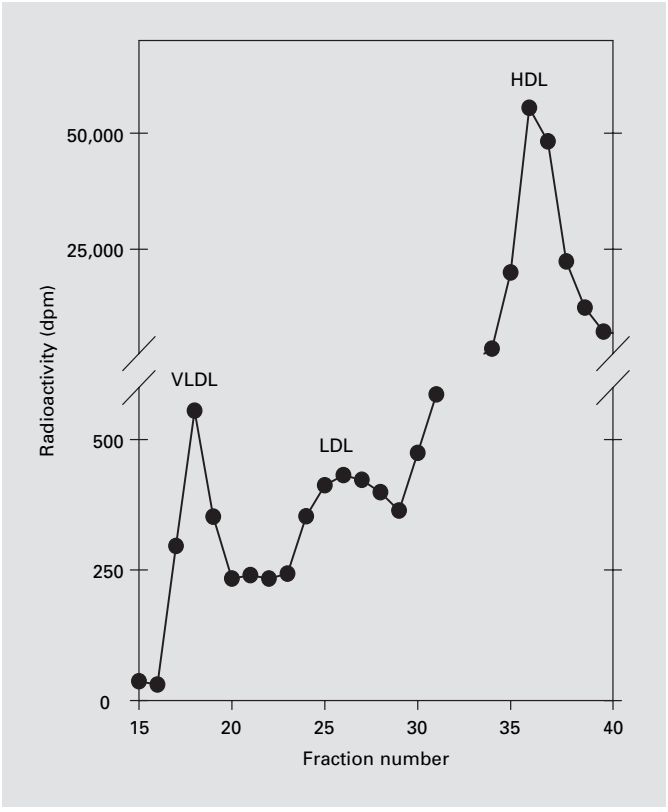



**Fig. 3.** $^3$H-allicin distribution in lipoproteins. LDL-R–/– mouse plasma was incubated with $^3$H-allicin. Lipoproteins were separated by size exclusion chromatography using a Superose-6 column. Radioactivity in each fraction was measured by a β-counter.

can be explained by the interaction between $Cu^{2+}$ and LDL, resulting in an increase in anisotropy and broadening of the ESR signal, which is characteristic for paramagnetic ion-biopolymer interactions [41]. The observed decrease in $Cu^{2+}$ ESR signal amplitude in the presence of LDL can also be explained by the reduction in $Cu^{2+}$ into $Cu^{1+}$, which is its diamagnetic form. However, in the case of LDL preincubated with allicin, we did not observe a pronounced decrease in peak intensity of the ESR $Cu^{2+}$ signal (fig. 5). In the control experiments, allicin itself did not affect the ESR signal of $Cu^{2+}$ (data not shown).

This result suggests that free SH groups of apoB-100 can participate in binding/reduction of $Cu^{2+}$ ions. The capability of allicin to penetrate through biological membranes and effectively modify the deeply buried SH groups of proteins was reported earlier [42, 43]. To check this idea, SH-specific MTS-SL was incubated with LDL. MTS-SL offers superior properties for the modification of free protein thiols (including membrane-bound pro-

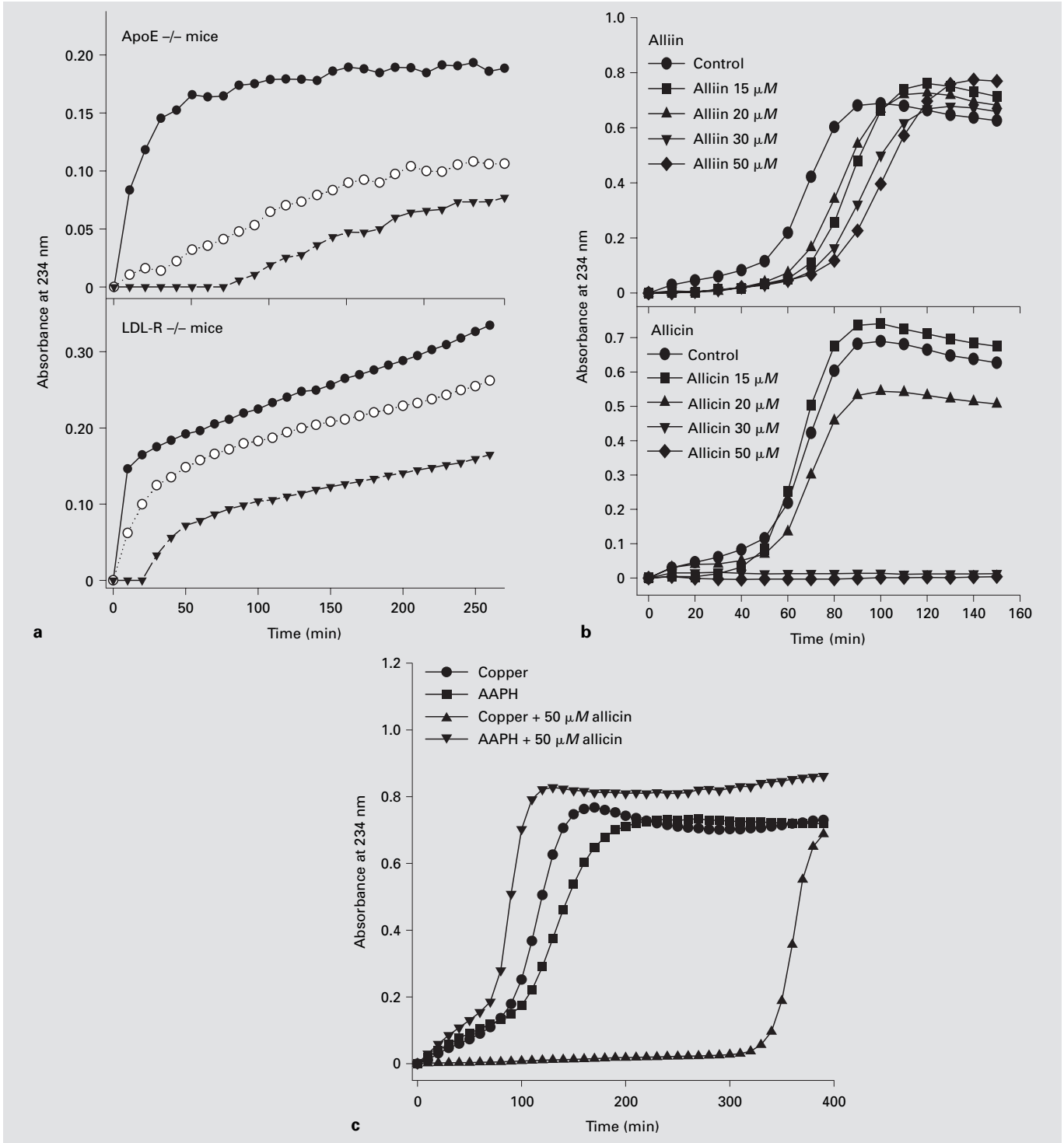



**Fig. 4.** The effect of allicin on LDL susceptibility to copper and AAPH-induced oxidation. **a** Ex vivo kinetics of LDL oxidation in apoE–/– and LDL-R–/– mice were examined. LDL (d = 1.063, top fraction) from fasting mice was isolated from pooled plasma taken from mice fed an atherogenic diet (8 weeks for apoE–/– mice and 6 weeks for LDL-R–/– mice) and treated with 3 (○) and 9 (▼) mg/kg body weight allicin or PBS (●). LDL (50 μg/ml) was incubated with 15 μ*M* $CuSO_4$ and conjugated diene formation was measured at 234 nm. **b** In vitro kinetics of LDL oxidation. LDL was isolated from the blood of a healthy volunteer as described in 'Materials and Methods'. LDL was incubated in PBS with or without allicin or alliin for 15 min. **c** The effect of allicin on copper and AAPH-induced LDL oxidation. Human LDL was oxidized with two radical generators: copper and AAPH.

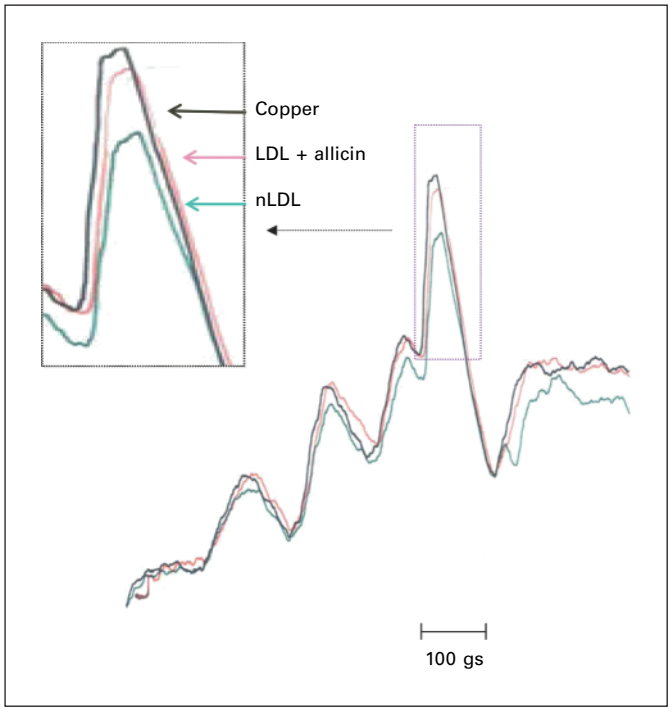



**Fig. 5.** The effect of allicin on LDL-copper complex formation. ESR spectra of $Cu^{2+}$ (0.1 m*M*) in the presence of nLDL and LDL pretreated with allicin (LDL + allicin) (2 mg/ml).

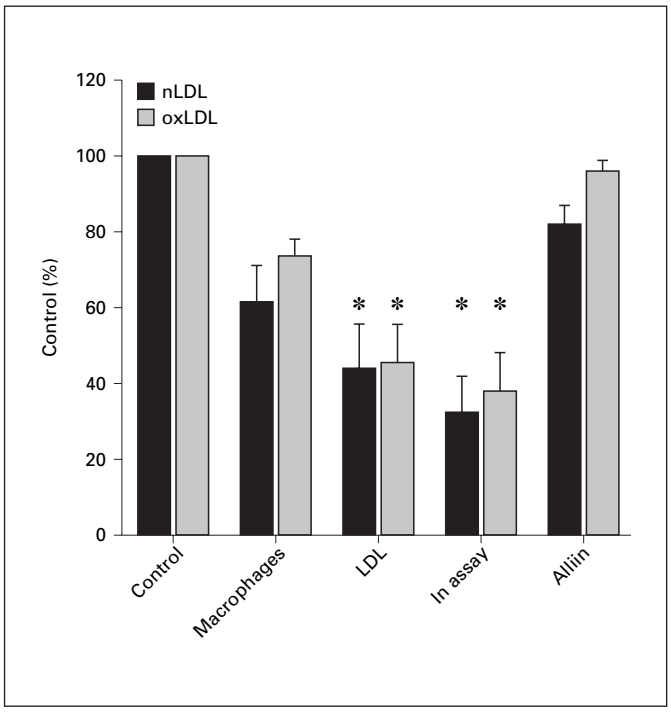



**Fig. 6.** The effect of allicin on LDL degradation in isolated mouse macrophages. $^{125}$I-nLDL and $^{125}$I-oxLDL were incubated with isolated mouse peritoneal macrophages. The effects of 50 μ*M* allicin or alliin on LDL degradation were assayed. Control = Untreated; macrophages = allicin added to macrophages and excess allicin washed out; LDL = allicin added to LDL and excess allicin dialyzed; in assay = allicin added to LDL and macrophage incubation mixture; alliin = alliin added to LDL and macrophage incubation mixture. The results represent the average of four experiments. * $p < 0.05$, significantly different from control.

teins) via a thiol-disulfide exchange reaction [44]. Like for allicin-treated LDL, a negligible decrease in peak intensity of the $Cu^{2+}$ ESR signal was observed (data not shown). Therefore, we conclude that both treatments reduced $Cu^{2+}$ binding to LDL in a similar way.

*Degradation of LDL by Isolated Peritoneal Macrophages*

As cellular oxidative stress influences scavenger receptor-mediated uptake of oxLDL, we tested the direct effect of allicin and alliin on $^{125}$I-LDL degradation by isolated peritoneal macrophages. Incubation of isolated macrophages with allicin reduced both nLDL and oxLDL degradation; however, this effect did not reach significance.

LDL and oxLDL pretreatment with allicin reduced LDL degradation, 56 and 54.5%, respectively ($p < 0.05$; ANOVA). In-assay addition of allicin (addition of allicin to the incubation media of macrophages and LDL) significantly inhibited both nLDL and oxLDL degradation by the isolated macrophages, 67.6 and 62%, respectively ($p < 0.05$; ANOVA), whereas alliin did not affect the process (fig. 6).

## Discussion

The present study demonstrates that daily administration of pure allicin to apoE–/– and LDL-R–/– mice significantly reduces the atherosclerotic lesion area in the aortic sinus. These results are consistent with our previous observation in C57BL/6 mice [24], showing a decrease in the early atherosclerosis lesion area in allicin-treated mice as compared with controls. Most studies investigated the effects of long-term feeding of garlic and garlic preparations on experimental atherosclerosis induced by a high-cholesterol diet in rabbits [21, 22]. Garlic preparation reduced the atherosclerotic lesion area of New Zealand rabbits following carotid artery deendothelialization [15, 17, 23].

In contrast to the positive results in rabbits, two garlic powder tablets (printanor and kyolic) did not affect the

atherosclerotic lesion area or its composition in APOE*3-Leiden transgenic mice [26]. By using pure allicin, we were able to investigate the effect of a well-defined component of garlic on atherosclerosis in the two well-known mouse models [45] and to seek for the possible mechanisms of its antiatherogenic activity.

Hyperlipidemia constitutes a major etiopathological factor for atherosclerosis [1–4].

In our study, we found that daily administration of pure allicin did not affect the total lipid levels; however, it altered the cholesterol distribution in lipoprotein particles from a proatherogenic profile toward an antiatherogenic profile, decreased VLDL and LDL, and increased the HDL cholesterol content in LDL-R–/– mice.

Several studies have suggested that garlic preparations may have beneficial effects on plasma cholesterol levels [12, 14–17, 20–24, 28], while other studies found no influence [12, 20–22, 26, 27]. We suggest that the composition and quantity of the sulfur components of different garlic preparations used in various studies could account in part for the inconsistent findings. The pharmacological activities of garlic are attributed to the thiosulfinate compounds, of which allicin is about 75% [26]. Lawson and Wang [46] determined the allicin-releasing ability of ten lots of allicin standardized garlic powder tablets, used in positive and negative clinical trails, under standardized gastrointestinal conditions. They demonstrated that the amount of allicin released correlates well with the success or failure of such tablets to lower the serum cholesterol value [46]. Therefore, the use of pure allicin to study the atheroprotective effect of garlic is reasonable.

Similar to our results, Espirito et al. [26], who also used well-characterized allicin, did not find any effect on plasma lipids; however, in their study, allicin did not influence the cholesterol lipoprotein profile. This dissimilarity may be due to the different mouse models used; in our study, the effect was detected in LDL-R–/– mice, while Espirito et al. [26, 27] used APOE*3-Leiden mice in their studies. Another factor that might affect the results is the route of allicin administration: daily oral administration by gavage in our study compared with supplementation in the drinking water. This is a critical concern, since allicin is a very reactive molecule which should be kept in dark and refrigerated in order to preserve its activity. Although the calculated doses in the studies were similar, approximately 3 mg/kg body weight per day, the actual dose in the studies by Espirito et al. [26, 27] might be affected by the exposure to light, temperature and food remnant in the water.

The antiatherogenic change in the lipoprotein profile following daily treatment with allicin seems insufficient to explain the approximately 60% reduction in the sinus lesion area. Therefore, we sought for other protective effects of allicin.

Oxidative modification of LDL is a key step in the atherosclerotic process [5–11].

Allicin administration to apoE–/– and LDL-R–/– mice strongly increased the resistance of plasma LDL to ex vivo oxidation by $Cu^{2+}$. Moreover, $Cu^{2+}$-induced oxidation of LDL in vitro showed that allicin protected LDL from oxidation better than its precursor alliin, even though both are antioxidants [13, 18, 20–22]. It is noteworthy that the effects of different garlic preparations, in which the exact allicin content is unknown, on $Cu^{2+}$-induced LDL oxidation are controversial [18, 19, 30]. Interestingly, we found that the protection of LDL by allicin from AAPH-induced oxidation is much less effective than its protection from $Cu^{2+}$-induced oxidation. Since oxidation of LDL by $CuSO_4$ is dependent on its binding to the lipoprotein and the LDL oxidation by AAPH is independent of such binding and acts through free radicals formation [3, 6, 7, 11], we assume that allicin may affect $Cu^{2+}$-induced oxidation by preventing its binding to the LDL particle.

According to Roland et al. [7], there are 38.6 ± 0.7 $Cu^{2+}$-binding sites per LDL particle, and nine of these binding sites are free thiols. Free thiols of cysteine residues are known to be a target for allicin [31]. By using radioactive-labeled allicin and ESR analysis, we demonstrated that allicin binds to LDL, VLDL and HDL and reduces $Cu^{2+}$ binding to LDL, respectively. We suggest that allicin may react with thiol groups on apoB in LDL, consequently interfering with $Cu^{2+}$ binding, and therefore inhibiting oxidation.

This hypothesis is supported by our results and those in previous studies, showing that N-ethylmaleimide and N-acetylcysteine, both high-affinity SH ligands, also inhibit $Cu^{2+}$ ligation to membrane proteins and LDL oxidation, respectively [47–49]. Moreover, we demonstrated that MTS-SL, known to bind to SH groups, inhibits $Cu^{2+}$ binding to LDL in a similar manner to that of allicin. Since apoB thiols reside in the hydrophobic environment of LDL [6, 7], the propensity of allicin to penetrate through biological membranes [42] may contribute to its lipoprotein-thiol-modifying potential.

Even though allicin inhibits $Cu^{2+}$-induced LDL oxidation in vitro and ex vivo, the relevance of this finding to LDL oxidation in vivo is not obvious [6, 10, 11]. However, the modification of LDL by allicin may affect other

processes in LDL metabolism. The oxidative modification of LDL and the subsequent uptake of oxLDL by macrophages, leading to foam cell formation, is a central step in atherogenesis [1–3, 8–10]. We show that uptake and degradation of both nLDL and oxLDL by isolated mouse peritoneal macrophages is inhibited by allicin-treated lipoproteins and/or macrophages. We hypothesize that the inhibition of LDL degradation by allicin is due to its binding to the free thiol groups on apoB or to free thiols on the scavenger receptors in macrophages, thus blocking the binding of LDL to macrophage receptors [49].

In this study, we concentrated on the effect of pure allicin, garlic-derived constituent, on the atherosclerotic process. We are aware that garlic contains a variety of organosulfur compounds, amino acids, vitamins and minerals [20–23] as well as allicin metabolites, which may also be involved in the antiatherogenic properties of allicin.

A critical question concerning the present study is whether orally administered allicin can reach the levels needed to affect biological processes in the plasma or within the arterial wall [12, 29]. In a previous study, allicin (orally administered) was not found in the blood, urine or stool [29, 50]. This issue is currently being tested in our laboratory by using the $^{3}$H-allicin. In a preliminary experiment, we were able to detect radioactivity in the blood and organs following oral administration of radiolabeled allicin (data not shown). It is noteworthy that allicin is very reactive and rapidly converted to its metabolites [51]. Therefore, we cannot exclude that most of the radioactivity detected resides in its metabolites, which might also confer atheroprotective effects.

In summary, by using a pure allicin preparation, we were able to show that allicin may affect atherosclerosis through several mechanisms such as LDL protection against oxidation, lipoprotein modification, inhibition of LDL uptake and degradation by macrophages.

## Acknowledgements

This work was supported by the Eduarda and Dr. Moshe Ishay Institute for the Study of the Effect of Natural Food and the Quality of Life and Human Health.

## References

1 Lusis AJ: Atherosclerosis. Nature 2000;407:233–241.
2 Glass CK, Witztum JL: Atherosclerosis: the road ahead. Cell 2001;104:503–516.
3 Stocker R, Keaney JF: Role of oxidative modifications in atherosclerosis. Physiol Rev 2004;84:1381–1478.
4 Parthasarathy S, Santanam N, Ramachandran S, Meilhac O: Oxidants and antioxidants in atherogenesis. An appraisal. J Lipid Res 1999;40:2143–2157.
5 Diaz MN, Frei B, Vita JA, Keaney JF: Antioxidants and atherosclerotic heart disease. N Engl J Med 1997;337:408–416.
6 Ferguson E, Singh RJ, Hogg N, Kalyanaraman B: The mechanism of apolipoprotein B-100 thiol depletion during oxidative modification of low density lipoprotein. Arch Biochem Biophys 1997;341:287–294.
7 Roland A, Patterson RA, Leake DS: Measurement of copper-binding sites on low density lipoprotein. Arterioscler Thromb Vasc Biol 2001;21:594–602.
8 Jessup W, Kritharides L: Metabolism of oxidized LDL by macrophages. Curr Opin Lipidol 2000;11:473–481.
9 Wang X, Greilberger J, Ledinski G, Kager G, Jurgens G: Binding and uptake of differently oxidized low density lipoprotein in mouse peritoneal macrophages and THP-1 macrophages: involvement of negative charges as well as oxidation-specific epitopes. J Cell Biochem 2001;81:557–569.
10 Chait A, Heinecke JW: Lipoprotein modification: cellular mechanisms. Curr Opin Lipidol 1994;5:365–370.
11 Burkitt MJ: A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: roles of lipid hydroperoxides, alpha-tocopherol, thiols, and ceruloplasmin. Arch Biochem Biophys 2001;394:117–135.
12 Ackermann R, Mulrow C, Ramirez G, Gardner C, Morbidoni L, Lawrence V: Garlic shows promise for improving some cardiovascular risk factors. Arch Intern Med 2001;161:813–824.
13 Imai J, Ide N, Nagae S, Moriguchi T, Matsuura H, Itakura Y: Antioxidant and radical scavenging effects of aged garlic extract and its constituents. Planta Med 1994;60:417–420.
14 Ali M, Al-Qattan KK, Al-Enezi F, Khanafer RM, Mustafa T: Effect of allicin from garlic powder on serum lipids and blood pressure in rats fed with a high cholesterol diet. Prostaglandins Leukot Essent Fatty Acids 2000;62:253–259.
15 Campbell JH, Efendy JL, Smith NJ, Campbell GR: Molecular basis by which garlic suppresses atherosclerosis. J Nutr 2001;131:1006S–1009S.
16 Eilat S, Oestraicher Y, Rabinkov A, Ohad D, Mirelman D, Battler A, Eldar M, Vered Z: Alteration of lipid profile in hyperlipidemic rabbits by allicin, an active constituent of garlic. Coronary Artery Dis 1995;6:985–990.
17 Kwon MJ, Song YS, Choi MS, Park SJ, Jeong KS, Song YO: Cholesteryl ester transfer protein activity and atherogenic parameters in rabbits supplemented with cholesterol and garlic powder. Life Sci 2003;72:2953–2964.
18 Munday JS, James KA, Fray LM, Kirkwood SW, Thompson KG: Daily supplementation with aged garlic extract, but not raw garlic, protects low density lipoprotein against in vitro oxidation. Atherosclerosis 1999;143:399–404.
19 Dillon SA, Burmi RS, Lowe GM, Billington D, Rahman K: Antioxidant properties of aged garlic extract: an in vitro study incorporating human low density lipoprotein. Life Sci 2003;72:2953–2964.
20 Brace LD: Cardiovascular benefits of garlic (*Allium sativum* L). J Cardiovasc Nurs 2002;16:33–49.
21 Orekhov AN, Grunwald J: Effects of garlic on atherosclerosis. Nutrition 1997;13:656–663.
22 Banerjee SK, Maulik SK: Effect of garlic on cardiovascular disorder: a review. Nutrition J 2002;1:1–14.
23 Efendy JL, Simmons DL, Campbell GR, Campbell JH: The effect of the aged garlic extract, 'Kyolic', on the development of experimental atherosclerosis. Atherosclerosis 1997;132:37–42.

24 Abramovitz D, Gavri S, Harats D, Levkovitz H, Mirelman D, Miron T, Eilat-Adar S, Rabinkov A, Wilchek M, Eldar M, Vered Z: Allicin-induced decrease in formation of fatty steaks (atherosclerosis) in mice fed on a cholesterol-rich diet. Coronary Artery Dis 1999;10: 515–519.
25 Prasad K, Laxdal VA, Yu M, Raney BL: Evaluation of hydroxyl radical-scavenging property of garlic. Mol Cell Biochem 1996;154:55–63.
26 Espirito Santo SM, van Vlijmen BJ, Buytenhek R, van Duyvenvoorde W, Havekes LM, Arnault I, Auger J, Princen HM: Well-characterized garlic-derived materials are not hypolipidemic in APOE*3-Leiden transgenic mice. J Nutr 2004;134:1500–1503.
27 Espirito Santo SM, van Vlijmen BJ, van Duyvenvoorde W, Offerman EH, Havekes LM, Arnault I, Auger J, Princen HM: Absence of an atheroprotective effect of the garlic powder printanor in APOE*3-Leiden transgenic mice. Atherosclerosis 2004;177:291–297.
28 Yeh YY, Liu L: Cholesterol-lowering effect of garlic extracts and organosulfur compounds: human and animal studies. J Nutr 2001;131: 989S–993S.
29 Lawson LD, Gardner CD: Composition, stability, and bioavailability of garlic products used in a clinical trail. J Agric Food Chem 2005;53:6254–6261.
30 Hsu CC, Huang CN, Hung YC, Yin MC: Five cysteine-containing compounds have antioxidative activity in Balb/cA mice. J Nutr 2004; 134:149–152.
31 Rabinkov A, Miron T, Konstantinovski L, Wilchek M, Mirelman D, Weiner L: The mode of action of allicin: trapping of radicals and interaction with thiol containing proteins. Biochim Biophys Acta 1998;1379:233–244.
32 Miron T, Bercovici T, Rabinkov A, Wilchek M, Mirelman D: [3H]Allicin: preparation and applications. Anal Biochem 2004;331:364–369.
33 Hatch FT: Practical methods for plasma lipoprotein analysis. Adv Lipid Res 1968;6:1–68.
34 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with Folin phenol reagent. J Biol Chem 1951;193:265–275.
35 Gieseg SP, Esterbauer H: Low density lipoprotein is saturable by pro-oxidant copper. FEBS Lett 1994;343:188–194.
36 Salacinski PR, McLean C, Sykes JE, Clement-Jones VV, Lowry PJ: Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent 1,3,4,6-tetrachloro-3 alpha,6 alpha-diphenyl glycoluril (Iodogen). Anal Biochem 1981;117:136–146.
37 Bellosta S, Mahley RW, Sanan DA, Murata J, Newland DL, Taylor JM, Pitas RE: Macrophage-specific expression of human apolipoprotein E reduces atherosclerosis in hypercholesterolemic apolipoprotein E-null mice. J Clin Invest 1995;96:2170–2179.
38 Goldstein JL, Basu SK, Brown MS: Receptor-mediated endocytosis of low density lipoprotein in cultured cells. Methods Enzymol 1983; 98:241–260.
39 Ishibashi S, Herz J, Maeda N, Goldstein JL, Brown MS: The two-receptor model of lipoprotein clearance: tests of the hypothesis in 'knockout' mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. Proc Natl Acad Sci USA 1994;91:4431–4435.
40 Kruczala K, Schlick S: Interaction of ionomers and polyelectrolytes with divalent transition metal cations ($Cu^{2+}$ and $VO^{2+}$): a study by electron spin resonance (ESR) spectroscopy and viscosimetry. Polym Mat Sci Eng 1999;79: 475–476.
41 Millard CB, Shnyrov VL, Newstead S, Shin I, Roth E, Silman I, Weiner L: Stabilization of a metastable state of *Torpedo californica* acetylcholinesterase by chemical chaperones. Protein Sci 2003;12:2337–2347.
42 Miron T, Rabinkov A, Mirelman D, Wilchek M, Weiner L: The mode of action of allicin: its ready permeability through phospholipid membranes may contribute to its biological activity. Biochim Biophys Acta 2000;1463:20–30.
43 Eliash T, Weiner L, Ottolenghi M, Sheves M: Specific binding sites for cations in bacteriorhodopsin. Biophys J 2001;81:1155–1162.
44 Aharoni A, Weiner L, Ottolenghi M, Sheves M: Bacteriorhodopsin experiences light-induced conformational alterations in nonisomerizable C(13) = C(14) pigments. A study with EPR. J Biol Chem 2000;275:21010–21016.
45 Veniant MM, Withycombe S, Young SG: Lipoprotein size and atherosclerosis susceptibility in Apoe(–/–) and LDL-R(–/–) mice. Arterioscler Thromb Vasc Biol 2001;21:1567–1570.
46 Lawson DL, Wang ZJ: Allicin release under simulated gastrointestinal conditions from garlic powder tablets employed in clinical trials on serum cholesterol. Planta Med 2001;67:13–18.
47 Lebedev VS, Volodina LA, Fedorov I: Analysis of the binding site of copper with intact and *Escherichia coli* bacteria modified by N-ethylmaleimide by ESR. Biofizika 1991;36:91–96.
48 Hong SY, Yang JO, Lee EY, Lee ZW: Effects of N-acetyl-*L*-cystein and glutathion on antioxidant status of human serum and 3T3 fibroblasts. J Korean Med Sci 2003;18:649–654.
49 Gruarin P, Sitia R, Alessio M: Formation of one or more intrachain disulphide bonds is required for the intracellular processing and transport of CD36. Biochem J 1997;328:635–642.
50 Lawson LD, Wang ZJ: Allicin and allicin-derived garlic compounds increase breath acetone through allyl methyl sulfide: use in measuring allicin bioavailability. J Agric Food Chem 2005;53:1974–1983.
51 Rosen RT, Hiserodt RD, Fukuda EK, Ruiz RJ, Zhou Z, Lech J, Rosen SL, Hartman TG: Determination of allicin, S-allylcysteine and volatile metabolites of garlic in breath, plasma or simulated gastric fluids. J Nutr 2001;131: 968S–971S.