Inhibition of Sterol 4α-Methyl Oxidase Is the Principal Mechanism by Which Garlic Decreases Cholesterol Synthesis

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ABSTRACT  Clinical and experimental evidence indicates that garlic ingestion lowers blood cholesterol levels, and treatment of cells in culture with garlic and garlic-derived compounds inhibits cholesterol synthesis. To identify the principal site of inhibition in the cholesterologenic pathway and the active components of garlic, cultured hepatoma cells were treated with aqueous garlic extract or its chemical derivatives, and radiolabeled cholesterol intermediates were identified and quantified. Garlic extract reduced cholesterol synthesis by up to 75% without evidence of cellular toxicity. Levels of squalene and 2,3-oxidosqualene were not altered by garlic, indicating that the site of inhibition was downstream of lanosterol synthesis, and identical results were obtained with 14C-acetate and 14C-mevalonate, confirming that 3-hydroxy-3-methylglutaryl-CoA reductase activity was not affected in these short-term studies. Several methylsterols that accumulated in the presence of garlic were identified by coupled gas chromatography–mass spectrometry as 4,4′-dimethylzymosterol and a possible metabolite of 4-methylzymosterol; both are substrates for sterol 4α-methyl oxidase, pointing to this enzyme as the principal site of inhibition in the cholesterologenic pathway by garlic. Of 9 garlic-derived compounds tested for their ability to inhibit cholesterol synthesis, only diallyl disulfide, diallyl trisulfide, and allyl mercaptan proved inhibitory, each yielding a pattern of sterol accumulation identical with that obtained with garlic extract. These results indicate that compounds containing an allyl-disulfide or allyl-sulfhydryl group are most likely responsible for the inhibition of cholesterol synthesis by garlic and that this inhibition is likely mediated at sterol 4α-methyl oxidase.  J. Nutr. 136: 759S–764S, 2006.

KEY WORDS: • garlic • cholesterol synthesis • sterol 4α-methyl oxidase • diallyl disulfide • lanosterol

Garlic is regarded with much interest by the general public as a means to safely reduce blood cholesterol levels. Indeed, several clinical trials and meta-analyses support the ability of garlic to reduce blood cholesterol, although the decrease is typically modest (1–3). Although the mechanism by which garlic reduces cholesterol levels has not been established, studies with garlic extracts have shown that garlic compounds inhibit cholesterol synthesis in cultured hepatocytes, in liver homogenates, and in cultured hepatoma cells (4–7) and that this inhibition occurs in a dose-dependent manner that is not related to cellular toxicity. In several studies the use of 14C-mevalonate instead of acetate prevented the decrease in cholesterol synthesis (5,8). This suggests that garlic decreased 3-hydroxy-3-methylglutaryl (HMG)5-CoA reductase activity, the second and regulated step in cholesterol synthesis. Other enzymes in the pathway were not examined, although Gebhardt et al. (5,9,10) reported that higher concentrations of extract, as well as allin-derived compounds, led to the accumulation of lanosterol, dihydrolanosterol, and 7-dehydrocholesterol, suggesting the inhibition of later steps in cholesterol synthesis.

Abbreviations used: DMEM, Dulbecco’s modified medium; HMG, 3-hydroxy-3-methylglutaryl.
Garlic is rich in sulfur-containing compounds, principally S-allylcysteine and allin, the latter of which is rapidly metabolized when garlic is crushed and allinase is released. The highly reactive sulfenic acid that is formed from allin condenses to allicin, which then rapidly recombines to various di- and trisulfides, depending on conditions. Ultimately these compounds are believed to yield allyl mercaptaand allyl methyl sulfide, which can react with cellular components or be eliminated on the breath. The organosulfur compounds formed in garlic are highly reactive with other sulfhydryl compounds, including cysteines found in proteins, and it is likely that the chemical modification of enzyme-sulfhydryls is responsible for the purported therapeutic effects of garlic. The question of which compounds are most important to the therapeutic effects of garlic remains unresolved, although several studies have shown that the diallyl disulfides, allyl mercaptaand S-alk(en)yl cysteines are effective inhibitors of cholesterol synthesis in cells (6–8,10). Similarly, the enzyme targets that mediate the effects of garlic have not been identified.

The present studies were undertaken to identify the cholesterolgenic enzyme or enzymes inhibited by garlic and the active principles therein. Our studies with hepatoma cells in which cholesterol and intermediates are radiolabeled and identified by coupled gas chromatography–mass spectrometry reveal that garlic causes the accumulation of sterol 4α-methyl oxidase substrates and that an allyl disulfide or allyl sulfhydryl group is necessary for inhibition by garlic-derived compounds.

**MATERIALS AND METHODS**

**Chemicals.** Dulbecco’s modified medium (DMEM), penicillin-streptomycin-glutamine (×100), fetal bovine serum, and trypsin were purchased from Invitrogen. Diallyl disulfide, diallyl trisulfide, allyl mercaptan, alllylsulfide, lactate dehydrogenase, pyruvate, NADH, Triton X100, cholesterol, ketoconazole, squalene, and zymosterol were purchased from Sigma Chemical Co. Zymosterol NADH, Triton X100, cholesterol, dihydrosterol, lathosterol, and zymosterol were identified by cochromatography of authentic standards visualized by iodine vapor and quantified by electronic autoradiography (Packard Instant Imager). Further confirmation of the identity of these and unknown steroids was obtained by scraping the corresponding region of nonradiolabeled samples into chloroform:methanol (2:1), derivatizing the samples with trimethylsilyland, and submitting them to gas-chromatographic separation on a Trace gas chromatograph with a DB-5ms column with helium carrier gas, followed by ion-trap mass spectrometry on a Thermofinnigan PolarisQ at the University of Kentucky Mass Spectrometry Facility.

**Determination of squalene, 2,3-oxidosqualene, and lanosterol synthesis.** For the determination of squalene, 2,3-oxidosqualene, and lanosterol synthesis, cells were incubated as described above for cholesterol synthesis with the inclusion of 60 μmol/L terbinfine, an inhibitor of squalene monoxygenase (for the determination of squalene), or 0.3 mmol/L AMO 1618, an inhibitor of oxidosqualene cyclase (for the determination of 2,3-oxidosqualene), or 10 μmol/L ketoconazole, an inhibitor of lanosterol demethylase (for the determination of lanosterol). Lipids were saponified by the addition of 0.5 mL of 10% methanolic potassium methoxide and incubated at 80°C for 1 h. For the determination of squalene and 2,3-oxidosqualene, the neutral lipids were extracted into 5 mL of petroleum ether; the solvent was removed by centrifugal evaporation, and the samples were resuspended in 50 μL of petroleum ether and resolved by silica thin-layer chromatography in 5% ethyl acetate in hexane. Lanosterol was determined as described for cholesterol. Authentic standards for squalene and lanosterol were visualized by iodine-vapor staining; 2,3-oxidosqualene was confirmed by cochromatography of the product of 14C-squalene conversion to 2,3-oxidosqualene by purified recombinant squalene monoxygenase (11). Further confirmation of these products was obtained by scraping the corresponding region of nonradiolabeled samples into chloroform:methanol (2:1) and submitting them to mass spectrometric analysis as described above.

**RESULTS**

Treatment of McARH7777 rat hepatoma cells with an aqueous garlic extract (8.5 g/L) reduced the incorporation of 14C-acetate into cholesterol over a 3-h time period by ≤75% without evidence of cellular toxicity (Fig. 1). At 7.5 g/L, garlic extract caused a marked elevation in lactate dehydrogenase activity in the medium, indicating the release of this enzyme from cells, and a significant loss of cell viability as measured by trypan blue exclusion. The ability of the extract to inhibit cholesterol synthesis at lower concentrations without toxicity suggested that 1 or more enzymes in the cholesterolgenic pathway were inhibited by garlic components.

Earlier studies had suggested that garlic inhibits cholesterol synthesis by reducing HMG-CoA reductase activity (5.8–10,12). To evaluate this possibility, squalene and 2,3-oxidosqualene synthesis was monitored in the presence of garlic extract, with the use of both 14C-acetate and 14C-mevalonate as substrates. Squalene and 2,3-oxidosqualene are the last 2 nonsterol intermediates in the cholesterolgenic pathway; as shown in Figure 2, the labeling of these intermediates was not affected by treatment with garlic extract. Moreover, cholesterol
labeling from $^{14}$C-mevalonate was decreased in the presence of garlic extract (Fig. 2B), indicating a site of inhibition downstream of HMG-CoA reductase. Together, these results indicate that the inhibition of cholesterol synthesis by garlic in these short-term studies is mediated at or beyond lanosterol demethylation.

Thin-layer chromatographic analysis of radiolabeled sterols from cells treated with garlic extract revealed the presence of several bands with a mobility characteristic of methylsterols (Fig. 3A). Treatment of cells with ketoconazole, an inhibitor of lanosterol demethylation, caused the accumulation of a prominent band (labeled “a” in Fig. 3A), which comigrated with authentic lanosterol. The identity of this sterol as lanosterol was confirmed by gas chromatographic analysis of the eluted sample (Fig. 3B) with comparison with authentic lanosterol, and by subsequent mass spectrometric analysis (data not shown). Although band “a” from the garlic-treated cells has a mobility apparently identical with that of lanosterol, gas chromatographic analysis of this product did not reveal the presence of lanosterol (Fig. 3B, center panel). In this sample, 2 prominent peaks were evident, with elution times of 23.23 min and 24.22 min. Mass spectrometric analysis revealed that the earlier peak corresponds most closely to 4-methyllanosterol (Fig. 3C), whereas the latter peak could not be identified. Despite repeated assays, lanosterol could not be shown to be present in garlic-treated samples. Gas chromatographic analysis of band “b” from the garlic-treated cells revealed 2 peaks eluting at 23.23 min and 24.88 min. The later peak was identified by mass spectrometry as 4,4‘-dimethylzymosterol (Fig. 3D), the first substrate for sterol 4α-methyl oxidase. The presence of 2 4-methyl, 14-demethylated sterols in the garlic-treated cells, and the absence of lanosterol, strongly suggests that garlic extract acts downstream of lanosterol demethylation to inhibit sterol 4α-methyl oxidase.

To identify the active principle(s) of garlic extract, 9 garlic-derived compounds were tested for their ability to inhibit cholesterol synthesis in hepatoma cells. Of these compounds, only diallyl disulfide, diallyl trisulfide, and allyl mercaptan were effective inhibitors of cholesterol synthesis at the micromolar level, and each yielded an inhibitory pattern identical with that of garlic extract (Fig. 4). Cholesterol synthesis was similarly reduced with $^{14}$C-mevalonate, and 2,3-oxidosqualene synthesis remained unaffected by these compounds, indicating that all compounds acted downstream of lanosterol synthesis (data not shown). Gas chromatographic–mass spectrometric analysis of the methylsterols that accumulated in the presence of diallyl disulfide yielded results identical with those obtained with garlic extract. Alliin and allyl methylsulfide were without effect on cholesterol synthesis, whereas S-allylcysteine and S-ethylcysteine reduced cholesterol synthesis by 10–20% but required considerably higher concentrations (4 mmol/L; data not shown). S-methylcysteine and S-propylcysteine were ineffective at concentrations ≥4 mmol/L. The estimated IC₅₀ for diallyl disulfide was 15 µmol/L, with maximal inhibition of ~80% at 200 µmol/L, where toxicity became evident (Fig. 5). The estimated IC₅₀ for diallyl trisulfide was somewhat higher, 40 µmol/L, and maximal inhibition reached only ~55% because of the appearance of toxicity at 100 µmol/L. Allyl mercaptan was the least potent inhibitor, with an IC₅₀ of ~200 µmol/L, but toxicity was not evident at concentrations ~750 µmol/L. Although these compounds appeared to fully replicate the inhibition obtained with garlic extract, the possibility that less abundant garlic derivatives, including allicin, contribute to the inhibition cannot be excluded. The similarity of the inhibitory pattern with all 3 agents to that of garlic extract argues that inhibition of sterol 4α-methyl oxidase is the principal mechanism by which garlic reduces cholesterol synthesis.

**DISCUSSION**

Early studies on the inhibition of cholesterol synthesis by garlic indicated that inhibition of HMG-CoA reductase was the likely mechanism by which garlic acted, on the basis of the observation that feeding garlic extract to chickens lowered serum cholesterol and reduced hepatic HMG-CoA reductase activity (13) and that HMG-CoA reductase could be inhibited in vitro by garlic or garlic-derived compounds (12). In subsequent studies by Gebhardt et al (5,9), incubation of rat hepatocytes and human hepatoma cells with a reconstituted
garlic extract inhibited HMG-CoA reductase activity by ≤23%, although a much greater reduction was seen in cholesterol synthesis and was attributed to the inhibition of additional downstream enzymes. At very low garlic concentrations, only HMG-CoA reductase inhibition appeared relevant, given that the substitution of 14C-mevalonate for 14C-acetate reduced the inhibition of cholesterol synthesis, as shown in the upper tracing labeled “Ketoconazole” in panel B. Bands “a” and “b” were eluted from the cells incubated in the presence of garlic (2.5 g/L) and submitted to gas chromatographic separation, as shown in the middle and lower tracings (labeled “a” and “b”) in panel B. (B) Gas chromatographic profiles of bands “a” and “b”; sterols were identified by mass spectrometry for the peaks at 24.40 (lanosterol), 23.23 (4-methylathosterol, shown in panel C), and 24.88 (4,4-dimethylzymosterol, shown in panel D). Other peaks did not correspond to sterols or could not be identified. The autoradiogram in (A) was merged from 2 images for clarity of presentation.

Our studies do not reveal an effect of garlic extract on HMG-CoA reductase, although a small reduction in activity cannot be excluded. The lack of change in squalene and 2,3-oxidosqualene labeling over a range of garlic concentrations that reduces cholesterol synthesis by ≤75% argues that inhibition of 1 or more enzymes downstream of HMG-CoA reductase must predominate at higher concentrations of garlic, a conclusion also reached by Gebhardt (5,9,10). In the studies of Liu and Yeh (7,8) relatively high concentrations of the S-alk(en)yl cysteines were needed to decrease cholesterol synthesis; the maximum inhibition achieved with S-allylcysteine was only 50% at a concentration of 4 mmol/L, yielding an IC₅₀ of 0.61 mmol/L.
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At this concentration we obtained a maximum of 10% inhibition with both $^{14}$C-acetate and $^{14}$C-mevalonate, arguing against a specific effect on HMG-CoA reductase. It should be noted, however, that Liu and Yeh used freshly isolated rat hepatocytes, whereas we used cultured rat hepatoma cells; differences between hepatocytes and hepatoma cells, as well as the medium and culture conditions may explain the different results.

Gebhardt concluded that higher concentrations of garlic and garlic-derived compounds inhibit lanosterol demethylase (5,9,10) on the basis of the accumulation of a radiolabeled band on thin-layer chromatography with the mobility of lanosterol. Our mass spectrometric analysis of this product and a second radiolabeled band with lower mobility was unable to demonstrate the presence of lanosterol but instead identified 4,4-dimethylzymosterol and 4-methylathosterol, a putative metabolite of 4-methyldymostanol. 4,4-Dimethylzymosterol and 4-methylathosterol are substrates for sterol 4α-methyl oxidase, an enzyme downstream of lanosterol demethylase that is known to be sensitive to sulfhydryl reagents (14). Moreover, ketoconazole, an inhibitor of lanosterol demethylase, yielded a different pattern of sterol intermediates both in our study (Fig. 3) and in Gebhardt’s report (5), lending support to our conclusion that sterol 4α-methyl oxidase, rather than lanosterol demethylase, is the most sensitive target of garlic inhibition.

Of 9 garlic-derived organosulfur compounds examined in the present study, only diallyl disulfide, diallyl trisulfide, and allyl mercaptan were inhibitory to cholesterol synthesis, each yielding a pattern of sterol accumulation identical with that obtained with garlic extract. Diallyl disulfide has previously been shown to inhibit HMG-CoA reductase in microsomes (12) and cholesterol synthesis in liver homogenates (4), primary hepatocytes (7,10,15), and hepatoma cells (6); diallyl trisulfide was similarly found to be effective in primary hepatocytes (7) and hepatoma cells (6), although cell toxicity was generally greater with the trisulfide, as found in the present study. Allyl mercaptan, the least potent inhibitor in our study, was similarly found to be 10–15% as effective as diallyl disulfide in hepatocyte culture (10,15) and hepatoma cells (6,16). Other garlic-derived compounds found to be effective inhibitors of cholesterol synthesis without overt toxicity include ajoene and methyl ajoene, allicin, 1,3-vinyl dithin (4,9,10), and some S-alk(en)yl cysteines (7,8,17). Excluding the alk(en)yl cysteines and the cyclic 1,3-vinyl dithin, all the inhibitory compounds share an allyl (or vinyl) group adjacent to a disulfide or sulphydryl group. Garlic compounds found not to be effective inhibitors lack this allyl-disulfide or allyl-sulfhydryl group and include alliin, S-methylcysteine, methylcysteine sulfoxide, propylcysteine sulfoxide, diallyl sulfide, dipropyl sulfide, and allyl methyl sulfide (4,5,7,9,15). Alliin, methylallyl sulfide, and several alk(en)yl cysteines (S-allylcysteine, S-methylcysteine, S-ethylcysteine, and S-propylcysteine) were shown to be ineffective in the present study.

The alk(en)yl cysteines S-allylcysteine, S-ethylcysteine, and S-propylcysteine appear to be unique in that they do not conform to the allyl disulfide/sulphydryl rule. Liu and Yeh (8) have shown that incubation of hepatocytes with S-allyl-, S-ethyl-, and S-propyl-cysteine lowers microsomal HMG-CoA reductase activity by 30–40% without changing enzyme mRNA or protein levels. This lower activity was attributed to an increase in the amount of phosphorylated (inactivated) enzyme in cells incubated with the alk(en)yl cysteines and additionally to an increase in sulphydryl oxidation in HMG-CoA reductase in the presence of S-allylcysteine. Although the S-alk(en)yl cysteines did not inhibit cholesterol synthesis in the present study, this laboratory has previously found S-allylcysteine to inhibit squalene monoxygenase, a downstream enzyme in the cholesteroligenic pathway, with an IC$_{50}$ of 110 μmol/L (18); S-methyl-, S-ethyl-, and S-propyl-cysteine, each of which lacks the allyl moiety, were not inhibitory to this enzyme.
Conjugation of the alk(en)yl cysteines to glutamate or acetate to form the γ-glutamyl and N-acetyl conjugates reduces their inhibitory potency (7), suggesting that there is something unique about the alk(en)yl cysteines that enhances their ability to downregulate HMG-CoA reductase activity; nonetheless, it should be noted that the concentrations of these organosulfur compounds needed to reduce HMG-CoA reductase activity in hepatocytes by 50% approaches the millimolar range (0.58–0.72 mmol/L).

Inhibition of cholesterol synthesis is thought to be a principal mechanism by which garlic lowers blood cholesterol, although other mechanisms may also be important. Indeed, there are very few studies on the effect of garlic on cholesterol synthesis in whole animals, and those early studies were limited to documenting a decrease in HMG-CoA reductase activity (13,19). Given that HMG-CoA reductase is down-regulated by isoprenoid and sterol intermediates (20–22), it can be expected that inhibition of a downstream cholesterolgenic enzyme will result in the accumulation of 1 or more intermediates that may feed back to decrease HMG-CoA reductase activity. Our conclusion that garlic inhibits sterol 4α-methyl oxidase is in accord with this view, given that 4-demethylated sterols, including lanosterol and dimethylzymosterol, have been shown to strongly promote the degradation of HMG-CoA reductase via an Insig-mediated pathway (22) (Fig. 6). Further studies are needed to determine whether garlic-derived organosulfur compounds inhibit purified sterol 4α-methyl oxidase and whether garlic effectively inhibits this enzyme in vivo.

**LITERATURE CITED**


**FIGURE 6** Feedback inhibition in the cholesterologenic pathway by garlic derivatives. A mechanism by which garlic compounds may down-regulate HMG-CoA reductase through inhibition of sterol 4α-methyl oxidase is illustrated. Only selected cholesterol intermediates are shown.