INHIBITION OF CHOLESTEROL, FATTY ACID, AND TRIGLYCERIDE BIOSYNTHESSES BY ORGANOSULFUR COMPOUNDS DERIVED FROM GARLIC IN PRIMARY CULTURES OF RAT HEPATOCYTES

A Thesis in
Nutrition
by
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ABSTRACT

The lipid-lowering effect of garlic has been shown in both animal and human studies. However, the active ingredients responsible for the lipid-lowering effect of garlic are poorly understood and the underlying mechanisms of garlic action are not fully known. Therefore, the present studies were conducted to identify the active compounds of garlic on cholesterol, fatty acid, and triglyceride syntheses and to elucidate the possible mechanisms underlying the inhibition of lipid synthesis by the compounds of garlic in primary cultures of rat hepatocytes.

The first objective of the thesis was to establish the inhibitory potency of organosulfur compounds of garlic on cholesterol biosynthesis. Cultured rat hepatocytes were treated with $[^{14}\text{C}]{\text{acetate}}$ as substrate for cholesterol synthesis in the presence or absence of test compounds at 0.05 to 4.0 mmol/L. Eleven water-soluble and six lipid-soluble compounds of garlic were tested. Among water-soluble compounds, three S-alk(en)yl cysteines, i.e., S-allyl-cysteine (SAC), S-ethyl cysteine (SEC), and S-propyl cysteine (SPC), inhibited $[^{14}\text{C}]{\text{acetate}}$ incorporation into cholesterol in a concentration-dependent manner achieving 42% to 55% maximal inhibition. Three $\gamma$-glutamyl S-alk(en)yl cysteines, i.e., $\gamma$-Glutamyl-S-allyl cysteine (GSAC), $\gamma$-glutamyl-S-methyl cysteine (GSMC), and $\gamma$-glutamyl-S-propyl cysteine (GSPC) were less potent, exerting only 16% to 29% maximal inhibition. S-allyl mercaptocysteine (SAMC), a disulfur-containing compound, inhibited the $[^{14}\text{C}]{\text{acetate}}$ incorporation into cholesterol by 7% to 17% at 0.05 and 0.5 mmol/L, but diminished the incorporation into cholesterol at 2.0 and 4.0 mmol/L. Alliin, S-allyl-N-acetyl cysteine (SANC), S-allylsulfonyl alanine (SASA), and S-methyl cysteine (SMC) had no effect on
cholesterol synthesis. Of the lipid-soluble compounds, diallyl disulfide (DADS), diallyl trisulfide (DATS), and dipropyl disulfide (DPDS) depressed cholesterol synthesis by 10% to 25% at low concentrations (≤ 0.5 mmol/L), and abolished the synthesis at high concentrations (≥ 1.0 mmol/L). Diallyl sulfide, dipropyl sulfide, and methyl allyl sulfide slightly inhibited [14C]acetate incorporation into cholesterol only at high concentrations. The complete inhibition of cholesterol synthesis by DADS, DATS, and DPDS was associated with extensive cytotoxicity as indicated by marked increase in cellular lactate dehydrogenase (LDH) release. There was no apparent increase in LDH release by water-soluble compounds except SAMC, which also abolished cholesterol synthesis. A comparison of cytotoxicity, maximal inhibition, and IC50 (concentration required for 50% of maximal inhibition) of various compounds suggests that water-soluble compounds of garlic may hold greater potential than lipid-soluble compounds in inhibiting cholesterol synthesis, which in turn may reduce plasma concentration of cholesterol. SAC, SEC, and SPC are the most potent inhibitors of cholesterol synthesis.

The second objective of the thesis was to determine the effects of water-soluble organosulfur compounds on triglyceride synthesis, fatty acid synthesis, and the activities of important lipogenic enzymes including fatty acid synthase (FAS) and glucose-6-phosphate dehydrogenase (G6PDH). When incubated with cultured hepatocytes, SAC and SPC decreased [14C]acetate incorporation into triglyceride in a concentration-dependent fashion (from 0.05 to 4.0 mmol/L), achieving maximal inhibitions of 43% and 51% at 4.0 mmol/L, respectively. The rate of [14C]acetate incorporation into phospholipids was depressed to a similar extent by SAC and SPC. SPC, SAC, SEC, and GSMC decreased [14C]acetate incorporation into fatty acid synthesis by 81%, 59%, 35%, and 40%, respectively, at 2.0-4.0
mmol/L concentrations. Alliin, GSAC, GSPC, SANC, SASA, and SMC had no effect on fatty acid synthesis. The activities of lipogenic enzymes, i.e., FAS and G6PDH were measured in cultured hepatocytes treated with the inhibitors. The activity of FAS in cells treated with SAC and SPC at the concentration of 4.0 mmol/L was 32% and 27% lower than that of non-treated cells, respectively. Neither SAC nor SPC affected G6PDH activity. The results indicate that SAC, SEC, and SPC inhibit triglyceride biosynthesis in cultured rat hepatocytes. More important, the study suggest that these S-alk(en)yl cysteines of garlic reduce triglyceride synthesis by decreasing de novo fatty acid synthesis resulting from the inhibition on FAS.

The third objective of the thesis was to examine the regulatory mechanisms underlying the inhibition of cholesterol synthesis by S-alk(en)yl cysteines. The treatment of cultured rat hepatocytes with S-alk(en)yl cysteines (i.e., SAC, SEC, and SPC), inhibited cholesterol synthesis from \[^{14}C\]acetate but not from \[^{14}C\]mevalonate, suggesting that the point of regulation is at 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase. Consistent with this notion, the activity of HMG-CoA reductase in the cells treated with SAC, SEC, and SPC was 30% to 40% lower than that of non-treatment group. Abundance of HMG-CoA reductase mRNA and the amount of the enzyme protein were not altered by the sulfur compounds. The expressed (E) and total (T) activities of HMG-CoA reductase were determined and the ratios of E/T were used to estimate the phosphorylation state of the enzyme. SAC, SEC, and SPC reduced the ratios of E/T by 18% to 29% resulting primarily from decreased expressed activity. The results suggest that organosulfur compounds of garlic decrease the activity of HMG-CoA reductase by phosphorylation but not by alteration in gene expression of the enzyme. In addition, the activity of HMG-CoA reductase was
measured at a lower dithiothreitol concentration and preincubation of microsomes with phosphatase to test the involvement of thiol redox status. Among three S-alk(en)yl cysteines, SAC was the only compound found to suppress the activity of the enzyme. Thus, SAC appears to inhibit HMG-CoA reductase activity by not only phosphorylating the enzyme, but also increasing sulfhydryl oxidation of the enzyme. The results strongly indicate that S-alk(en)yl cysteines modify the activity of HMG-CoA reductase at the posttranslational level.

In summary, the results of the present studies indicate that water-soluble organosulfur compounds are the most important compounds in inhibiting hepatocyte cholesterol synthesis. Lipid-soluble sulfur compounds, on the other hand, may not be the major compounds in the reduction of cholesterol synthesis because of their cytotoxicity. Among the organosulfur compounds, water-soluble compounds SAC, SEC, and SPC are the most potent inhibitors of cholesterol synthesis. The inhibition of cholesterol synthesis by S-alk(en)yl cysteines results from the decreased activity of HMG-CoA reductase. The suppressed activity of HMG-CoA reductase by S-alk(en)yl cysteines is attributed to the inactivation of the enzyme by phosphorylation but not gene expression of the enzyme. SAC may also increase sulfhydryl oxidation of the enzyme. In addition, S-alk(en)yl cysteines inhibit triglyceride synthesis due in part to reduced de novo fatty acid synthesis. These results have added to our understanding of the active constituents of garlic responsible for the lipid-lowering effects and the underlying mechanisms.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4S</td>
<td>Scandinavian Simvastatin Survival Study</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl CoA carboxylase</td>
</tr>
<tr>
<td>AGE</td>
<td>aged garlic extract</td>
</tr>
<tr>
<td>AHA</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BHQ</td>
<td>black hole quencher</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>CARE</td>
<td>Cholesterol and Recurrent Events</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesterol ester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CPF</td>
<td>Cyp7α promoter binding factor</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DADS</td>
<td>diallyl disulfide</td>
</tr>
<tr>
<td>DAS</td>
<td>diallyl sulfide</td>
</tr>
<tr>
<td>DATS</td>
<td>diallyl trisulfide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPDS</td>
<td>dipropyl disulfide</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DPS</td>
<td>dipropyl sulfide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>E/T ratio</td>
<td>the ratio of expressed to total activity of HMG-CoA reductase</td>
</tr>
<tr>
<td>FAM</td>
<td>carboxyfluorescein-aminohexyl amidite;</td>
</tr>
<tr>
<td>FAS</td>
<td>fatty acid synthase</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>G6PDH</td>
<td>glucose-6-phosphate dehydrogenase</td>
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<tr>
<td>GSAC</td>
<td>γ-glutamyl-S-allyl cysteine</td>
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<tr>
<td>GSMC</td>
<td>γ-glutamyl-S-methyl cysteine</td>
</tr>
<tr>
<td>GSPC</td>
<td>γ-glutamyl-S-propyl cysteine</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl-CoA</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>concentration required for 50% of maximal inhibition</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate density lipoprotein</td>
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<tr>
<td>LCAT</td>
<td>lecithin-cholesterol acyltransferase</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LIPID</td>
<td>Long-term Intervention with Pravastatin in Ischaemic Disease</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL receptor-related protein</td>
</tr>
<tr>
<td>MAS</td>
<td>methyl allyl sulfide</td>
</tr>
<tr>
<td>MUFA</td>
<td>monounsaturated fatty acids</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCEP</td>
<td>National Cholesterol Education Program</td>
</tr>
<tr>
<td>PPB</td>
<td>potassium phosphate buffer</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SAC</td>
<td>S-allyl cysteine</td>
</tr>
<tr>
<td>SAMC</td>
<td>S-allylmercaptocystene</td>
</tr>
<tr>
<td>SANC</td>
<td>S-allyl-N-acetyl cysteine</td>
</tr>
<tr>
<td>SASA</td>
<td>S-allylsulfonyl alanine</td>
</tr>
<tr>
<td>SEC</td>
<td>S-ethyl cysteine</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SMC</td>
<td>S-methyl cysteine</td>
</tr>
<tr>
<td>SRBI</td>
<td>scavenger receptor BI</td>
</tr>
<tr>
<td>SPC</td>
<td>S-propyl cysteine</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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</table>
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INTRODUCTION

Heart disease is a major public health concern and the leading cause of death in the United States (1). Increased blood levels of total cholesterol, particularly low density lipoprotein (LDL) cholesterol are risk factors for coronary heart disease (CHD, 2). There is substantial evidence that lowering total and LDL-cholesterol levels reduce the incidence of CHD and coronary death (2). Many primary and secondary prevention trials have proven the benefits of cholesterol-lowering (3). Aside from cholesterol, elevated levels of plasma triglyceride have been associated with an increased risk of cardiovascular disease (4-6). A recent meta analysis revealed that increased triglyceride level is a risk factor for cardiovascular disease independent of high density lipoprotein (HDL) cholesterol level (7). Studies in both human and animal models have shown that triglyceride-rich lipoproteins such as intermediate density lipoprotein (IDL) and very low density lipoprotein (VLDL) are related to the extent and severity of atherosclerosis (8). Lowering triglyceride levels may reduce the risk of CHD (9).

One of the strategies to lower blood lipids is lifestyle intervention such as diet modifications and controlling body weight (10). For diet modification, diets with low saturated fat (< 7% of total calories) and cholesterol (< 200 mg/day) are recommended for people who want to lower their blood lipids and reduce the risk for CHD (11). Dietary supplements such as soy protein and garlic have also been found to reduce blood lipid levels (12, 13).

It has been reported that garlic decreased plasma lipids, especially total cholesterol and LDL cholesterol in both humans and animals (14-16). Meta-analyses have revealed that
plasma cholesterol concentration is reduced 9% to 12% in subjects treated with garlic as compared to a placebo group (17, 18). Garlic also reduces blood triglyceride levels (19, 20). In contrast to these reports, several recent clinical trials failed to confirm the cholesterol-lowering effect of garlic (21-24). Although the reason for the observed discrepancy is not readily known, it is important to point out that the different garlic preparations were used in the clinical trials such as garlic powder, garlic oil, and aged garlic extracts. The composition of sulfur compounds is different among the different garlic preparations. Raw garlic and garlic powder contain lipid-soluble thiosulfinates such as allicin, thiosulfinate transformation products, and sulfides (25). Garlic oil mainly contains sulfides (25). Among these compounds, allicin and vinyl dithiins have been suggested as potential lipid lowering agents (25). In addition, garlic in general and aged garlic extract in particular contain significant amounts of water-soluble organosulfur compounds including S-alk(en)yl cysteines and γ-glutamyl-S-alk(en)yl cysteines. Thus, active constituents and/or the amounts of active constituents may be critical to produce the desired effect. However, the active constituents responsible for the lipid-lowering effect of garlic have not been fully defined. Furthermore, the underlying mechanisms of cholesterol-lowering action of garlic have not been elucidated (26). The present studies were conducted to identify the active ingredients responsible for the lipid-lowering effect of garlic and to explore the possible mechanisms underlying the effect of garlic in primary cultures of rat hepatocytes. The results indicate that water-soluble compounds, particularly SAC, SEC, and SPC, were the most potent inhibitors of lipid synthesis in cultured rat hepatocytes. The inhibition of S-alk(en)yl cysteines on cholesterol synthesis stems from the decreased activity of HMG-CoA reductase by increased phosphorylation of the enzyme. SAC may decrease HMG-CoA reductase activity by
increasing sulfhydryl oxidation of the enzyme as well. The reduction of triglyceride synthesis by S-alk(en)yl cysteines is a result of decreased fatty acid synthesis due to the suppressed activity of FAS.


CHAPTER 1

LITERATURE REVIEW
1.1 CORONARY HEART DISEASE

Coronary heart disease (CHD) remains a major public health problem in the United States and other developed countries. The principal underlying cause of CHD is atherosclerosis. Atherosclerosis is primarily a chronic inflammatory disease, which proceeds in multiple steps. The first step involves injury of the endothelium leading to endothelial dysfunction. As a response to the injury, monocytes, macrophages, and T-lymphocytes accumulate in the intima forming the earliest atherosclerotic lesion. The macrophages gradually ingest lipid and become foam cells, and under the influence of various growth factors smooth muscle cells proliferate and secrete extracellular matrix. This process continues for years and leads to the formation of fibrous plaques which consist of a fibrous cap overlaying a lipid-rich core. In the latter stages, the core of the lesion becomes necrotic and eventually calcified. Atherosclerotic plaques narrow and may totally obstruct the lumen of the affected artery. Furthermore, if the plaque ruptures or ulcerates, the exposed content causes platelets to adhere and aggregate, thereby creating a thrombus over the site of plaque. Therefore, atherosclerosis finally results in ischemia causing angina pectoris, myocardial infarction (MI) and other coronary events (1, 2).

Endothelial dysfunction, on the other hand, may result from elevated and modified low-density lipoprotein (LDL), free radicals caused by cigarette smoking, hypertension, elevated plasma homocysteine level, infectious microorganisms, and combinations of the above (2).
1.1.1 Prevalence, incidence, and mortality of coronary heart disease in the United States

In the United States, approximately 12,400,000 people alive today have a history of CHD including MI, angina pectoris or both (3). It is estimated that 1,100,000 Americans will have new or recurrent coronary events including MI or fatal CHD in 2001. CHD was the single largest killer of Americans and caused 459,841 deaths in U.S. in 1998, i.e., 1 of every 5 deaths (3). CHD mortality among man and women in 1998 was about the same (total male deaths-233,374, total female deaths-226,467). CHD is the leading cause of premature, permanent disability in the U.S. labor force accounting for 19% of disability allowances by the Social Security Administration (3).

1.1.2 Risk factors for CHD

Population-based studies such as the Framingham Heart Study have given rise to the concept that specific factors, both genetic and environmental, modifiable and non-modifiable, increase the risk of developing cardiovascular disease (CVD) (4). Hypercholesterolemia, hypertension, and cigarette smoking are the major modifiable risk factors (5). Other important modifiable risk factors include lowered high-density lipoprotein (HDL) cholesterol level, diabetes mellitus, obesity, and physical inactivity. More recently, evidence has accumulated to implicate a possible association of increased risk of CHD with chronic inflammation, increased blood homocysteine, and elevated plasma triglyceride (4-6). Family history, male sex and advancing age are nonmodifiable risk factors (5). Sections from 1.1.2.1 to 1.1.2.6 are a brief review focused on the major modifiable risk factors and some of the recently identified ones.
1.1.2.1 Cholesterol

Evidence accumulated from observational epidemiological studies over the past several decades strongly supports the contention that total serum cholesterol is a major predictive factor in the development of CHD (7, 8). Data culled from various observations including a study of seven countries (9), population-based investigations within the United States such as the Framingham Heart study (10, 11), and prospective epidemiological investigations such as the Pooling Project (12), have established the important role of total serum cholesterol in the development of CHD in a clear, dose-related and temporal manner. The findings from the Multiple Risk Factor Intervention Trial (MRFIT) provide additional support for the relationship of increased total serum cholesterol levels to the risk of dying from CHD (13). Among the approximately 356,000 males between the ages of 35 and 57 years who were screened for this trial, a continuous and graded relationship between the level of serum cholesterol at the time of screening and deaths from CHD over a six-year follow-up period was observed.

Low-density lipoprotein and high-density lipoprotein cholesterols have emerged as the most important lipoprotein complexes related to the onset of clinical events resulting from coronary atherosclerosis. A number of epidemiological studies have shown that the levels of LDL cholesterol are positively related to the incidence of CHD, while the levels of HDL cholesterol are inversely related to the incidence of CHD, i.e., high levels of HDL cholesterol have a protective effect and are associated with a reduced risk of CHD (7, 14, 15). Such relations are independent of the usual coronary risk factors such as cigarette use and hypertension. A 1% increase in LDL value is associated with an increase of slightly more than a 2% in coronary artery disease (CAD) over 6 years; a 1% decrease in HDL value is
associated with a 3% to 4% increase in CAD. Even at total cholesterol levels less than 200 mg/dl, low HDL levels are associated with increased MI rates in both men and women (16).

LDL, which may be modified by oxidation and glycation (in diabetes mellitus), is a major cause of injury to the endothelium and underlying smooth muscle. When LDL particles become trapped in an artery, they can undergo progressive oxidation and be internalized by macrophages through the scavenger receptors on the surfaces of these cells. The internalization leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters, resulting in the formation of foam cells. In addition to its ability to injure these cells, modified LDL is chemotactic for other monocytes, and can up-regulate the expression of genes for macrophage colony-stimulating factor and monocyte chemotactic protein. Thus, it may expand the inflammatory responses by stimulating the replication of monocyte-derived macrophages and the entry of new monocytes into lesions (2).

Atheroprotective mechanisms of HDL may be involved in different stages of the multiple-step process of atherosclerosis such as the inhibition of adhesion molecule induction, prevention of LDL modification, and removal of excess cholesterol by reverse cholesterol transport (17, 18).

1.1.2.2 Blood pressure

Elevation of either systolic or diastolic blood pressure has been shown to be predictive of the subsequent risk of CHD, with hypertension consistently and independently established as a major risk factor for CHD in both national and international studies (7, 19). A meta-analysis, which examined data from nine large prospective observational studies, revealed a direct and continuous relationship between the increasing levels of diastolic blood
pressure and the risk of CHD events (20). Hypertension is a powerful independent contributor to cardiovascular morbidity and mortality, on average conferring a threefold increase in risk at all ages and in both sexes (21).

1.1.2.3 Cigarette smoking

Cigarette smoking has been consistently related in a dose-responsive manner to the development of fatal and nonfatal CHD events (22-24). Data from the Framingham Heart Study have shown that the consumption of cigarettes is clearly related to the risk of MI, sudden cardiac death, and CHD mortality in both men and women (25). The risk of cardiovascular morbidity and mortality is greatly affected by cigarette smoking. For every 10 cigarettes per day there is an incremental increase in cardiovascular mortality of 18% in men and 31% in women (21).

Currently, approximate 25,870,000 men (27.1%) and 22,830,000 women (22.2%) are smokers among Americans age 18 and older (26). Among whites, 26.5% of men and 23.6% of women smoke; and among blacks, 29.0% of men and 21.3% of women smoke (3). An estimated 4,100,000 adolescents ages 12 to 17 are smokers (26).

1.1.2.4 Homocysteine

The association between elevated levels of homocysteine and cardiovascular disease was first reported by McCully (27). McCully described progressive arterial disease, often resulting in death from thrombosis in children with homocystinuria. Homocystinuria is a rare genetic disease caused primarily by a deficiency of cystathionine β-synthase resulting in severely elevated plasma levels of homocysteine (> 200 µmol/L) (4, 28). Cystathionine β-
synthase is an enzyme catalyzing the formation of cystathionine from homocysteine in the transsulfuration pathway of homocysteine catabolism (4, Figure 1.1). Recently, attention has been shifted from homocystinuria to investigation of the more common condition of moderately elevated levels of homocysteine (29). Clinical and epidemiological studies have shown that the increased plasma concentration of homocysteine is independently associated with an increased risk for atherosclerosis, thrombosis, and premature CVD (30). A recent study reported that a higher mortality (25%) in CHD patients with homocysteine levels above 15 µmol/L than that (4%) in patients with homocysteine levels below 9 µmol/L (31). The exact mechanism by which homocysteine exerts its atherothrombotic action is still unclear. Accumulating evidence suggests that hyperhomocysteinemia leads to endothelial injury and dysfunction, stimulates the proliferation of vascular smooth muscle cells, promotes thrombosis by increased generation of thrombin, interferes with nitric oxide-dependent vasodilation, and increases adhesion of monocytes to endothelium (32, 33).

Plasma homocysteine concentration is determined by its synthesis and catabolism. Several B vitamins play important regulatory roles in homocysteine metabolism (34). Vitamin B_6_ is the cofactor of cystathionine β-synthase that irreversibly converts homocysteine to cystathionine. Vitamin B_{12} is the cofactor of methionine synthase which remethylates homocysteine to methionine. Folate in the 5-methyltetrahydrofolate form donates the methyl group in this reaction (34, Figure 1.1).

Plasma folate, vitamin B_6_ and vitamin B_{12} levels are inversely related to plasma homocysteine level (35). Intakes of these vitamins, especially folate are also inversely related to the plasma homocysteine concentration (34, 35). Supplementation of the diet with folate alone or combination of folate, vitamin B_6_, and vitamin B_{12}, and/or consumption of folate-
rich food such as vegetables and citrus fruit has been shown to reduce homocysteine levels (36-39). Epidemiological studies have shown that low dietary intakes of folate, vitamin B₆, and vitamin B₁₂ or low blood concentrations of these vitamins are associated with increased incidence of CAD (40-42). Currently, there has been no randomized, controlled trial of homocysteine-lowering vitamins with hard CVD endpoints (43). However, results from intervention trials suggest that B vitamins have a protective effect on CVD development (44-46). Large-scale, double-blind randomized trials are necessary to confirm the benefits of B vitamin supplementation on CVD risk (45, 47).
Figure 1.1 Homocysteine metabolism
Enzymes: 1) 5,10-methylenetetrahydrofolate; 2) methionine synthase; 3) S-adenosylmethionine synthase; 4) S-adenosylhomocysteine hydrolase; 5) cystathionine β-synthase; 6) betaine homocysteine methyltransferase; 7) glycine N-methyltransferase; 8) serine hydroxymethylase; 9) cystathionase. (adapted from Oparil and Oberman (4))
1.1.2.5 Triglyceride

Elevated levels of plasma triglyceride have been associated with an increased risk of CVD (48, 49). However, whether hypertriglyceridemia is an independent factor was disputed until the publications of Austin et al. (50, 51). The meta-analysis by Austin et al. revealed that elevated triglycerides were independently associated with coronary artery disease incidence, i.e., increased triglyceride level was a risk factor for cardiovascular disease, independent of HDL cholesterol level (50, 51). This result suggests that triglyceride-rich lipoproteins such as IDL and VLDL are independently atherogenic. Studies in both human and animal models have shown that triglyceride-rich lipoproteins are related to the extent and severity of atherosclerosis (52).

1.1.2.6 Chronic inflammation

Chronic inflammation has been proposed to be a risk factor for CHD (53). Plasma levels of C-reactive protein, which is a marker for systemic inflammation, are elevated in patients with acute MI or acute ischemia (54, 55). CHD has also been associated with a variety of gram-negative bacterial infections such as Helicobacter pylori, and herpesvirus infections, particularly cytomegalovirus (CMV). There is, however, no proof of the possible causative role of infection (1, 56).

1.1.3 Management of CHD risk factors—lipid-lowering

A number of studies have shown the reduction of morbidity and mortality of CHD with the control of risk factors (57) such as cessation of smoking (58-60), lowering of blood pressure (61, 62), dietary supplementation of folate and vitamin B₆ to decrease plasma
homocysteine levels (63), and lipid-lowering. Sections 1.1.3.1 to 1.1.3.3 are a brief review on lipid- and cholesterol-lowering benefits and strategies.

### 1.1.3.1 Benefits of cholesterol-lowering

Many primary and secondary prevention trials have been conducted to establish and verify the beneficial effects of lowering cholesterol levels in the blood. There is now a widespread agreement that an elevated level of LDL-cholesterol is a major risk factor for the development of CHD; LDL is therefore the primary target of cholesterol-lowering (64). Primary prevention generally encompasses the effort to modify the risk factors or to prevent their development with the aim of delaying or preventing the new-onset CHD, and the secondary prevention involves a therapy to reduce the recurrent CHD events and to decrease the coronary mortality in patients with established CHD (65).

#### 1.1.3.1.1 Primary prevention trials

Early primary prevention trials such as the World Health Organization clofibrate trial (66), the Lipid Research Clinics coronary primary prevention trial (67, 68), and the Helsinki gemfibrozil trial (69) all produced an approximately 10% decrease in total cholesterol levels. Despite such modest reductions in serum cholesterol levels, these therapies yielded substantial reductions in acute coronary events compared with controls. Taken together, the findings from these trials support the concept that a 1% lowering of cholesterol levels reduces CHD risk by 2% in middle-aged people.

The results of a major primary prevention trial—the West of Scotland Coronary Prevention Study (WOSCOPS) were published in 1995 (70). This multicenter trial
randomized high-risk men with hypercholesterolemia and no history of CHD to pravastatin therapy or placebo. Pravastatin reduced total cholesterol by 20% and LDL-cholesterol by 26%. This therapy also reduced major coronary events by 31% and all-cause mortality by 22%.

Recently, another primary prevention trial, the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS, 71), compared the effects of lovastatin with placebo on the frequency of first major coronary events (unstable angina, MI, or sudden CHD death) in 5,608 men and 997 women who had average levels of total cholesterol and LDL-cholesterol (mean total cholesterol of 221 mg/dL, mean LDL cholesterol of 150 mg/dL). After an average follow-up of 5.2 years, lovastatin therapy was found to have reduced LDL-cholesterol levels by 25% and decreased major coronary events by 37%.

1.1.3.1.2 Secondary prevention trials

In early secondary prevention trials that employed different therapeutic regimens such as cholesterol-lowering diets and drugs, the degree of serum cholesterol lowering was only moderate, averaging about 10% (72). In most of these trials, a trend toward a reduction in recurrent CHD events was observed, but only in some of the trials was the reduction statistically significant (72). However, when data from all trials were pooled and analyzed as a single set, i.e., meta-analysis, the results revealed that recurrent CHD events and CHD mortality were significantly reduced by cholesterol-lowering therapy, with a strong trend toward a reduction in total mortality (73).
Several angiographic trials to test the effects of cholesterol-lowering therapies on progression and regression of atherosclerotic lesions have been reported (74). Most of these trials used therapies that produced marked reductions in cholesterol levels (72). Angiography revealed that cholesterol-lowering therapy compared with placebo usually slowed the progression of coronary lesions. In some instances, the treatment induced regression of the lesions (72). New coronary events (unstable angina and acute MI) were also markedly reduced (74).

Recently, three large secondary prevention trials using statin agents have been carried out: the Scandinavian Simvastatin Survival Study (4S, 75), Cholesterol and Recurrent Events (CARE, 76) trial, and the Long-term Intervention with Pravastatin in Ischaemic Disease (LIPID) study (77). Findings from 4S demonstrated that simvastatin therapy in patients with hypercholesterolemia and established CHD reduced total mortality by 30%, total deaths due to coronary events by 42%, and major coronary events by 34%. In the CARE trial, patients with CHD and average cholesterol levels (mean total cholesterol, 209 mg/dL; all below 240 mg/dL) were treated with pravastatin or placebo. Pravastatin therapy significantly reduced definite nonfatal MI and coronary death by 24%. The LIPID trial showed results similar to those of CARE, but LIPID additionally showed a reduction in total mortality with pravastatin in patients who had a broad range of initial cholesterol levels (155-271 mg/dL).

1.1.3.2 Benefits of management of other lipids

High plasma triglycerides are independent risk factors for CHD development (50). However, the role of serum triglyceride reduction in achieving clinical benefit is unclear and poorly documented. One randomized, double-blind, placebo-controlled trial indicated that the
The progression of coronary artery disease could be reduced with the reduction of triglyceride-rich lipoproteins (78). This trial provides strong evidence of the benefit of decreasing serum triglycerides.

A low level of serum HDL-cholesterol is a potent predictor of premature CHD (79). The Department of Veterans Affairs HDL Intervention Trial (VA-HIT) was the first major clinical trial specifically focusing on the treatment of low HDL cholesterol. This trial showed for the first time that lipid treatment, which had no effect on LDL cholesterol level but raised HDL cholesterol and lowered triglycerides, substantially reduced nonfatal MI or CHD death in patients with established CHD whose major lipid abnormality was a low level of HDL cholesterol (80).

1.1.3.3 Strategies to achieve the goal of lipid-lowering

There are two major strategies to lower blood lipids (81, 82). One strategy is lifestyle intervention including diet modifications, regular exercise, and controlling body weight. For diet modification, Step I or Step II diets should be followed for the people who want to lower their cholesterol. Step I and Step II diets are low in saturated fat and cholesterol (64). A meta-analysis from 37 dietary intervention studies demonstrated that Step I intervention lowered plasma total cholesterol 10%, LDL cholesterol 12%, and triglyceride 8% (83). Step II intervention lowered the same parameters by 13%, 16%, and 8%, respectively. HDL cholesterol was decreased by 7% in response to Step II but not to Step I dietary interventions (83). In addition, other dietary constituents such as soy protein and garlic (discussed in section 1.3.4.4) have been found to reduce blood lipid levels (84, 85).
The other strategy is pharmaceutical therapy in which lipid-lowering drugs are administered to reduce cholesterol. For people who have relatively high cholesterol, especially for patients with CHD, lifestyle intervention therapies may not be sufficient to achieve desired levels (86), and cholesterol-lowering drug therapies such as statins, bile acid resins, nicotinic acid, and fibrates are often needed in addition to non-pharmaceutical therapy (81, 87).

In patients with low HDL cholesterol levels, lifestyle intervention such as smoking cessation and weight loss may increase HDL cholesterol (88). Although elevated plasma triglyceride levels are independent risk factors for CHD (50), reduction of triglyceride levels in patients with hypertriglyceridemia is associated with an increase in HDL cholesterol levels (88).
1.2 LIPID METABOLISM

Cholesterol, fatty acids, and triglyceride are important lipids in the body (89, 90). Lipids are a major source of energy (triglyceride) and have many other functions such as the structure of membrane (cholesterol) and the control of the body’s homeostasis (prostaglandins and steroid hormones). However, imbalances of lipid metabolism can lead to some of the major clinical problems such as cardiovascular disease and obesity (11, 13, 90). Sections from 1.2.1 to 1.2.10 are a brief review of cholesterol metabolism, fatty acid and triglyceride biosyntheses, with special emphasis on their regulation.

1.2.1 Biosynthesis of cholesterol

All the carbon atoms in cholesterol are provided by acetyl-CoA, and the reducing equivalents are provided by reduced nicotinamide adenine dinucleotide phosphate (NADPH). The pathway is driven by the hydrolysis of the high-energy thioester bond of acetyl CoA and the terminal phosphate bond of ATP. Synthesis occurs in the cytoplasm, with the enzymes located in both the cytosol and the endoplasmic reticulum (ER) (89, 91, 92). Acetyl-CoA is primarily derived from pyruvate through glycolysis of carbohydrate (mainly glucose), amino acid oxidation, fatty acid oxidation, and ketone bodies (93-95).

The biosynthetic pathway of cholesterol is illustrated in Figure 1.2. This pathway involves a series of over 20 reactions (92). The major regulatory step in cholesterol synthesis is the conversion of HMG-CoA to mevalonate, catalyzed by HMG-CoA reductase (89). Other enzymes such as squalene synthase and squalene monooxygenase (formerly called squalene epoxidase) are also regulated at the level of gene expression by sterols (96, 97).
Lanosterol analogs decrease cholesterol biosynthesis by inhibiting lanosterol 14α-methyl demethylase (98, 99).

Acetyl CoA + Acetoacetyl CoA
HMG-CoA synthase ↓
  HMG-CoA
HMG-CoA reductase ↓
  Mevalonate
  ↓
  Mevalonate-PP*
  ↓
  Isopentenyl-PP
  ↓
  Geranyl-PP
  ↓
  Farnesyl-PP
Squalene Synthase ↓
  Squalene
Squalene monooxygenase ↓
  Squalene-2,3-epoxide
  ↓
  Lanosterol
  ↓
  Lanosterol 14α-methyl demethylase↓
  ↓
  Cholesterol

**Figure 1.2** Pathway of cholesterol synthesis * PP, pyrophosphate

1.2.2 Regulation of cholesterol synthesis

HMG-CoA reductase is the rate-limiting enzyme in cholesterol synthesis (100) which is the focus of this section. The regulation of HMG-CoA reductase may exert at the transcriptional or post-transcriptional level (101). Post-transcriptional regulation exerts at the levels of translation, protein degradation, and catalytic efficiency including phosphorylation and thiol redox status of the enzyme (100, 102).
1.2.2.1 Feedback inhibition

In animal cells, cholesterol is obtained from two sources: (1) endogenous synthesis (described in section 1.2.1); and (2) exogenously derived from plasma LDL, which enters the cell by receptor (LDL-receptor)-mediated endocytosis. When cholesterol is abundant in the cell, the activities of HMG-CoA synthase and HMG-CoA reductase are low, and LDL-receptor production is decreased, i.e., cholesterol exerts a feedback regulation of HMG-CoA synthase, HMG-CoA reductase, and LDL-receptor. Other sterols such as oxysterols have similar effects to that of cholesterol in inhibiting cholesterol synthesis (101).

Food deprivation results in marked reduction of cholesterogenesis in animals and moderate suppression to almost complete suppression in humans (103). Cholesterol feeding suppresses hepatic HMG-CoA reductase activity and cholesterol synthesis in animals (103, 104). In humans, dietary cholesterol has been shown to decrease or have no effects on cholesterol synthesis (103, 105). Polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) increase cholesterol synthesis although they lower plasma cholesterol concentrations (103). PUFA and MUFA enhance cholesterol removal from circulation by up-regulating LDL receptor, which could offset the increase of cholesterol synthesis and lead to the reduction of plasma cholesterol (103, 106). Stearic acid also increases cholesterol synthesis in animals and in cultured rat hepatocytes (107, 108). Stearic acid is a unique saturated fatty acid because it does not raise plasma cholesterol concentration (109). Human and animal studies have confirmed the “neutral” or hypocholesterolemic effect of dietary stearic acid (110). This effect could be explained by reduced cholesterol absorption and increased excretion of endogenous cholesterol (108).


1.2.2.2 Hormonal regulation

Animal studies demonstrated that hepatic cholesterol synthesis was decreased in the fasted rat, and increased upon refeeding (111). The regulation of cholesterol synthesis in fasting and refeeding is mainly mediated by glucagon and insulin through HMG-CoA reductase (102). HMG-CoA reductase exists in phosphorylated (inactive), and dephosphorylated (active) forms (112, 113). Glucagon stimulates phosphorylation (inactivation), and insulin promotes dephosphorylation, thus activating the enzyme and favoring cholesterol synthesis (112, 113). In addition, glucagon and insulin may alter the gene expression of HMG-CoA reductase (102).

1.2.2.3 Regulatory mechanisms of HMG-CoA reductase

Mammalian HMG-CoA reductase, a 97 kd glycoprotein, is an integral membrane protein of the endoplasmic reticulum (92, 114). The C-terminal two-thirds of the polypeptide chain extends into cytosol of the cell and contains the catalytic activity. The N-terminal one-third of the polypeptide chain, not required for catalytic activity, contains alternating hydrophobic and hydrophilic segments that traverse the membrane eight times (92, 114, 115).

1.2.2.3.1 Transcriptional control of HMG-CoA reductase

The induction and repression of the mRNA for HMG-CoA reductase are clearly observed in Chinese hamster ovary (CHO) cells that have been cultured in the presence and absence of sterols (101). In addition, the mRNAs for LDL receptor and HMG-CoA synthase show the same pattern as HMG-CoA reductase in response to sterols, i.e., these mRNAs are
coordinately regulated (92, 101). A series of studies using fusion genes have identified the sterol regulatory nucleotide sequence in the three genes, which is designated sterol regulatory element-1 (SRE-1). The synthase promoter also contains another related sequence, designated SRE-2. Recent studies have found several sterol regulatory element binding proteins (SREBPs), and these proteins might mediate the transcriptional regulation acting as transcription factors (92, 116).

**1.2.2.3.2 Posttranscriptional control of HMG-CoA reductase**

The post-transcriptional control of this enzyme is primarily regulated by non-sterol products (117). The mechanisms are both at the level of translation and through degradation of the enzyme (101). Studies have indicated that the N-terminal domain may be involved in the degradation of the enzyme because the truncated enzyme lacking all the membrane-spanning domains is localized to the cytosol and is quite stable (118).

**1.2.2.3.3 Phosphorylation of HMG-CoA reductase**

HMG-CoA reductase is also inactivated by phosphorylation (92). This reaction is catalyzed by a new member of protein kinase family, AMP-activated protein kinase (119). AMP-activated protein kinase may not play a role in end-product feedback control, but rather it comes into play when cellular ATP levels are depleted, thereby lowering the rate of cholesterol synthesis and preserving the energy stores of the cell (120).
1.2.2.3.4 Sulfhydryl oxidation of HMG-CoA reductase

The susceptibility of HMG-CoA reductase to inactivation by sulfhydryl oxidation has been well documented (121-124). Microsomes prepared with thiols possess an active HMG-CoA reductase, while microsomes prepared without thiols contain an inactive, latent enzyme (121). The oxidative inactivation is reversible in the presence of high concentrations of thiols such as dithioerythritol, dithiothreitol and glutathione (121, 122) and may involve the formation of an intramolecular protein-SS-protein disulfide (123).

1.2.3 Roles of liver and lipoproteins in cholesterol homeostasis

Liver plays a central role in the regulation of cholesterol homeostasis (125, 126). On one hand, liver is the only place for the net excretion of cholesterol from the body through bile including biliary cholesterol and bile acids which is discussed in 1.2.4 (125, 126). On the other hand, liver is the major organ in the body to regulate plasma lipid levels such as cholesterol and triglyceride (125-127). One aspect of plasma lipid regulation by liver is the synthesis of lipids, the assembly of VLDL from triglycerides, cholesterol, apolipoproteins and phospholipids, and secretion of VLDL to the circulation system (128, 129). The other aspect of plasma lipid regulation by liver is the uptake of lipids from blood through LDL-receptor, LDL receptor-related protein (LRP) and scavenger receptor BI (SRBI) associated with LDL, VLDL remnants, chylomicron remnants, and HDL (129).

1.2.3.1 Production of VLDL and LDL

VLDL is assembled from triglycerides, cholesterol, apolipoprotein B and phospholipids in liver and secreted into blood (128, 129). Triglyceride is the major lipid in
VLDL. In blood, VLDL interacts with lipoprotein lipase which is located on endothelial cell surfaces in peripheral tissues and hydrolyzes VLDL triglycerides. As lipolysis proceeds, VLDL particles become smaller and more dense (VLDL remnant) and are converted to IDL. The excess surface materials, mostly phospholipids and cholesterol are transferred to HDL (129, 130). The HDL interacts with the plasma enzyme lecithin-cholesterol acyltransferase (LCAT), which esterifies the excess cholesterol to cholesterol ester. The newly synthesized cholesterol ester is transferred back to IDL, and triglyceride in IDL is transferred to HDL through the action of plasma cholesterol ester transfer protein (CETP). The net result of the coupled lipolysis and exchange reactions is the replacement of most of triglyceride core of VLDL with cholesterol esters. The IDL undergoes a further conversion to LDL, which mainly contains cholesterol esters in the core and apolipoprotein B at the surface (129, 130). LDL is the major cholesterol carrier in plasma (129).

**1.2.3.2 Lipid uptake from circulation by liver**

Liver can take up lipids from lipoproteins in bloodstream through receptor-mediated pathway. The cellular metabolism of LDL particles primarily via the LDL receptor and other members of the LDL receptor family which process LDL via endocytic uptake and lysosomal degradation (131, 132). In lysosomes, cholesterol esters are hydrolyzed to cholesterol and fatty acids, apolipoprotein B is degraded to amino acids, and the degraded products are released to cytosol. But the LDL receptor escapes degradation and returns to the cell surface (130, 131). The clearance of LDL from plasma is mainly determined by the availability and activity of LDL receptors, and the liver is the most important organ for the activity of LDL.
receptors (127, 129). Other lipoproteins such as VLDL remnant and chylomicron remnant can also be taken up by LDL receptors and LRP (129).

HDL begins in the liver and small intestine as small protein-rich disk containing relatively little cholesterol and no cholesterol esters (129). The small cholesterol-poor HDL disks can adsorb free cholesterol from cell membranes and become nascent HDL₃ particles. Following the esterification of free cholesterol to cholesterol ester by LCAT and the movement of cholesterol ester from the surface to the core of the particle, HDL₃ particles continue to adsorb free cholesterol because the core of the spherical particles can accumulate more molecules of cholesterol ester than could be accommodated on the surface. The continued adsorption of free cholesterol enables HDL₃ to enlarge such that they can accommodate more phospholipids and apolipoproteins and form HDL₂. HDL₂ exchanges cholesterol ester for TG with apo B-containing lipoproteins such as IDL via CETP. HDL₂ can also deliver cholesterol to the liver by selective uptake of core cholesterol ester through SRBI (133). The transport of cholesterol from extrahepatic tissues to liver by HDL is so-called reverse cholesterol transport (129), which could be one of the atheroprotective mechanisms of HDL (18).

1.2.3.3 Regulation of plasma cholesterol concentration by diets

While a low-fat, high-carbohydrate diet effectively reduces plasma cholesterol concentrations, it also increases plasma TG concentrations and reduces HDL-cholesterol concentrations (134). There is evidence that the type of fat in diets has different effects on plasma cholesterol levels and hence the type of fat is also important as the amount of fat (127). High cholesterol and saturated fat diets increase plasma cholesterol levels, especially
LDL cholesterol. Cholesterol and saturated fat down-regulate the LDL receptor and inhibit the removal of LDL from the plasma by the liver. Saturated fat down-regulates the LDL receptor, especially when cholesterol is concurrently present in the diet (106). When excess cholesterol is delivered to the liver from the diet, the sterol in liver cell increases leading to a decrease in LDL receptor and an increase in cholesterol ester formation through acyl-CoA cholesterol acyltransferase (ACAT) (127). Polyunsaturated fatty acids and monounsaturated fatty acids up-regulate the LDL receptor and increase the uptake of LDL from plasma. In addition, PUFA and MUFA have been shown to depress synthesis of VLDL and the production of LDL (106).

Other dietary components such as soy protein, organosulfur compounds of garlic, and plant stanol have also been shown to decrease plasma cholesterol (135). Although the mechanisms of their lipid-lowering effects are not well defined, the inhibition of hepatic lipid synthesis, and the inhibition on the absorption of dietary and biliary cholesterol by these dietary constituents are some of the possible mechanisms (135, 136).

1.2.4 Synthesis of bile acid

The conversion of cholesterol to bile acids represents a major pathway for the excretion of cholesterol from the body (125). It is the major catabolic route for cholesterol metabolism, and only a smaller portion of cholesterol is converted to steroid hormones (137). Bile acids are synthesized in the liver by a multistep pathway in which hydroxyl groups are inserted at specific positions on the steroid structure, the double bond of the steroid nucleus is reduced, and the hydrocarbon chain is shortened by three carbons, introducing a carboxyl group at the end of the chain. The resulting compounds, cholate and chenodeoxycholate, are
The rate-limiting step of bile acid synthesis is the first reaction, in which cholesterol is converted to 7α-hydroxycholesterol catalyzed by cholesterol 7α-hydroxylase.

Before the bile acids leave the liver, they are conjugated with a molecule of either glycine or taurine to form bile salts. After conjugation, bile salts are secreted via the bile ducts and gallbladder into the lumen of the small intestine where they act as detergents to emulsify dietary lipid and fat-soluble vitamins (138). Bacteria in the intestine can remove glycine and taurine from bile salts. They can also convert some of the primary bile acids into secondary bile acids by removing a hydroxyl group, producing deoxycholate and lithocholate. Most of bile salts secreted into the intestine are reabsorbed and reused through enterohepatic circulation (125, 139).

1.2.5 Regulation of bile acid synthesis

The first step of bile acid synthesis catalyzed by cholesterol 7α-hydroxylase is the rate-limiting step (138). Cholesterol 7α-hydroxylase is a microsomal cytochrome P450 enzyme and is coded by the Cyp7α gene (138). This enzyme is activated in a feedforward manner by dietary cholesterol in some species such as rats and mice. It is repressed by products of the pathway such as cholate and chenodeoxycholate (138). Thus, bile salts returning to the liver from the intestine through enterohepatic circulation decrease bile acid synthesis (126). If the bile salts are prevented from returning to the liver, the activity of the enzyme increases. Therefore, the conversion of cholesterol to bile salt and its excretion increase (125, 126). This effect is the basis of bile acid resins, a therapeutical drug for treating hypercholesterolemia (125, 126).
Recently, progresses have been made in understanding these regulatory mechanisms. First, studies have shown that oxysterols such as 24-hydroxycholesterol and 24,25-epoxycholesterol increase the Cyp7α transcription by activation of the nuclear orphan receptor LXRα (140, 141). The LXRα response element has been found in the promoter region of Cyp7α gene (141). Second, another protein, named Cyp7α promoter binding factor (CPF), which is also a nuclear orphan receptor, has been reported to be a possible activator of Cyp7α transcription; CPF may be responsible for the liver-specific regulation (142). Third, bile acids such as chenodeoxycholate have been shown to suppress Cyp7α transcription by activation of FXR, another nuclear orphan receptor (143).

1.2.6 Biosynthesis of fatty acids

In mammals, fatty acid synthesis occurs primarily in liver and lactating mammary glands and in adipose tissue (144). The energy for the carbon-to-carbon condensations in fatty acid synthesis is supplied by the process of carboxylation and then decarboxylation of acetyl groups in the cytosol. The carboxylation of acetyl CoA to form malonyl CoA is catalyzed by acetyl CoA carboxylase (ACC), and requires ATP and biotin as the coenzyme (145). Carbons from acetyl CoA and malonyl-CoA are incorporated into the growing fatty acid chain in the cytosol, utilizing ATP and NADPH (145).

All of the reactions in the synthetic process are catalyzed by a multienzyme complex, the fatty acid synthase (FAS) (145).

The overall fatty acid synthesis pathway is:

\[ 8 \text{ Acetyl CoA} + 7\text{ATP} + 14\text{NADPH} + 14\text{H}^+ \rightarrow \]

Palmitate + 8\text{CoA} + 6\text{H}_2\text{O} + 7\text{ADP} + 7\text{Pi} + 14\text{NADP}^+ \]
1.2.7 Regulation of fatty acid synthesis

Control of fatty acid synthesis is accomplished mainly by regulation of the two committed enzymes of this process, ACC and FAS (146). ACC is rate-limiting in the short-term (minutes to hours) regulation, while FAS is rate-limiting in the long-term (hours to days) regulation (90).

ACC and FAS decrease during starvation and increase upon refeeding a high-carbohydrate, low-fat diet. Conversely, a high-fat diet, and particularly a diet rich in polyunsaturated fatty acids, suppresses fatty acid synthesis. The dietary effects are mediated principally via two hormones—insulin, which increases lipogenesis during feeding, and glucagon, which is responsible for inhibiting lipogenesis during starvation (146).

1.2.7.1 Regulation of ACC

ACC is regulated predominantly by covalent modification and allosteric mechanisms (90). Glucagon triggers phosphorylation of this enzyme and inactivates it, thereby slowing fatty acid synthesis (147). Phosphorylation of ACC is catalyzed by AMP-activated protein kinase, the same enzyme as in the phosphorylation of HMG-CoA reductase (92). Insulin causes dephosphorylation and activation of ACC (147). Palmitoyl CoA, the principal product of fatty acid synthesis acts as a feedback inhibitor of the enzyme, and citrate is an allosteric activator (147). In addition, ACC may also be regulated at the level of gene expression (147, 148).
ACC exists as two major isoforms, ACC\textsubscript{\alpha} and ACC\textsubscript{\beta} (147, 148). There is evidence that ACC\textsubscript{\alpha} plays an important role in the production of malonyl-CoA for fatty acid synthesis, whereas ACC\textsubscript{\beta} involves in the control of mitochondrial \(\beta\)-oxidation (147, 148).

**1.2.7.2 Regulation of FAS**

Active animal FAS is a homodimer with each peptide subunit about 260 kDa in size. Each subunit contains eight functional domains. Seven domains represent the enzyme activities required for fatty acid synthesis. The eighth domain represents the acyl carrier protein, the site to which the growing fatty acid is tethered during synthesis (90).

Unlike ACC, FAS is regulated predominantly at the transcriptional level (90). Paulauskis and Sul (149) showed that refeeding after fasting resulted in a great increase in FAS mRNA in mice. In a later work, the same authors observed that insulin induced FAS mRNA in livers of diabetic mice, whereas the increase seen by fasting/refeeding was abolished by administration of dibutyryl cAMP, which is the mediator of glucagon’s action (150). Polyunsaturated fatty acids suppress FAS gene transcription possibly mediated by SREBP and hence decrease fatty acid synthesis (151).

Studies have identified several response elements in the promoter of the FAS gene including Insulin Response Element (IRE) and PUFA response sequences (90, 151). These elements are located in the 2.1-kb region flanking the 5'-end of the FAS gene. The 2.1-kb region is sufficient for activation by dietary carbohydrate, insulin, and for deactivation by glucagon (152, 153).
1.2.8 Major source of the NADPH for hepatic cholesterol and fatty acid syntheses

The pentose phosphate pathway is the major NADPH provider for reductive biosynthesis, which contributes 50-75% of the NADPH used in hepatic lipid synthesis. Glucose-6-phosphate dehydrogenase (G6PDH) is the key regulatory enzyme in this pathway (154, 155). G6PDH, like ACC and FAS, is subject to tissue-specific regulation by hormones and nutrients. Recent studies have demonstrated some possible regulatory mechanisms such as gene transcription and mRNA stability (155). NADPH is also generated by malic enzyme in the process of citrate cleavage to regenerate acetyl-CoA in cytosol, which is the major source of NADPH in adipose tissue (156).

1.2.9 Triglyceride biosynthesis

Triglyceride synthesis begins with the activation of long-chain fatty acids to their CoA thioesters (157). Acyl-CoAs are then esterified to glycerol-3-phosphate to form phosphatidate. Phosphatidate is hydrolyzed to form diacylglycerol, which undergoes a final acylation step to form triglyceride (Figure 1.3, 157).

Triglycerides and glycerophospholipids share two precursors (acyl-CoA and glycerol-3-phosphate) and several enzymatic steps in their biosynthesis in animal tissues (157, 158). Glycerol-3-phosphate can arise from dihydroxyacetone phosphate during glycolysis and from glycerol by the action of glycerol kinase in liver and kidney (157, 158).
Fatty acid
\[ \text{ACS} \downarrow \]
Glycerol-3-Phosphate + Acyl-CoA
\[ \text{GPAT} \downarrow \]
Lysophosphatidate + Acyl-CoA
\[ \text{LPAAT} \downarrow \]
Phosphatidate
\[ \text{PPH} \downarrow \]
Diacylglycerol -> Glycerophospholipids
\[ \text{DGAT} \downarrow \]
Triglyceride

**Figure 1.3** Major routes of triglyceride synthesis.
ACS, acyl-CoA synthetase; DGAT, diacylglycerol acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; LPAAT, Lysophosphatidate acyl-CoA acyltransferase; PPH, phosphatidate phosphohydrolase.

### 1.2.10 Regulation of triglyceride biosynthesis

Low-fat, high-carbohydrate diets can increase plasma triglyceride levels (159). However, the mechanism is unclear. Recent studies indicate that the effect on plasma triglyceride by high-carbohydrate diets may be due to increased hepatic fatty acid synthesis, thus resulting in accelerated VLDL-triglyceride secretion (159, 160). High PUFA diets decrease TG synthesis (151). In addition, TG synthesis is also regulated by hormones similar to fatty acid synthesis. Insulin promotes TG synthesis and glucagon decreases it (157).
Phosphatidate phosphohydrolase (PPH), the enzyme catalyzed the dephosphorylation of phosphatidate to diacylglycerol is an important enzyme in glycerolipid metabolism (161). Regulation of PPH activity is thought to play a prominent role in controlling the rate of glycerolipid synthesis because its activity is low relative to other TG synthetic enzymes, and because diet and hormone-induced changes in its activity are more dramatic than changes in other enzymes (161, 162). Diacylglycerol acyltransferase (DGAT), the enzyme catalyzed the terminal and only committed step in triglyceride synthesis plays a fundamental role in triglyceride synthesis (163, 164). Recent studies indicate that mice lacking DGAT are viable and resistant to diet-induced obesity (165). Thus, this enzyme could be a new target for obesity treatment (164, 165).
1.3 GARLIC

Garlic has long been revered for its medicinal properties, as evidenced by ancient writings from Egypt, Greece, China, and India extolling its merits (166, 167). This reverence has evolved into serious scientific respect as a result of the emergence of data indicating that garlic may influence the risk of cardiovascular disease and cancer (166). Sections 1.3.1 to 1.3.4 are a brief discussion on garlic, focused on its chemistry and health benefits.

1.3.1 Botanical classification

In a new classification after embryologic and phytochemical examination, garlic is considered a member of the Alliaceae family, not the Liliaceae family as it is still identified in many textbooks and taxonomy manuals (168). Its botanical name is *Allium sativum* L. and it originated from central Asia (169, 170). Onion, leek and chive are examples of other *Allium* plants (168). Garlic is a hardy, low-growing perennial plant that bears small white flowers; bulky-like roots contain several bulbils (clove). The cloves are enclosed in a silky white or pink envelope/skin (171).

1.3.2 Chemistry of garlic

The water content of garlic (about 65%) is rather low compared to that of most fruits and vegetables (80-90%). Garlic consists of approximately 28% carbohydrate, mainly in the form of fructans, and 1.8% protein. Lipids comprise 0.1-0.2% of the fresh weight of garlic (172). The sulfur content of garlic is close to 1.0% of the dry weight (173). Its remarkably high content of sulfur and sulfur-related compounds has drawn considerable attention to garlic. Approximately 90% of the investigations on garlic’s compounds have focused on its sulfur compounds (172, 173).
1.3.2.1 Important discoveries of sulfur compounds of garlic

For centuries, the odor-causing components of garlic were thought to contain sulfur until 1844. In that year, the German scientist Wertheim discovered that steam distillation of crushed garlic produced a strong-smelling oil that consisted exclusively of organosulfur compounds. These compounds were later determined to be allyl sulfides. This was the first scientific demonstration that sulfur-containing compounds are responsible for the odor of garlic (172).

In 1944, Cavallito and Bailey isolated and identified the substance responsible for the antibacterial activity of crushed garlic clove as an oxygenated sulfur compound that possessed the odor of freshly cut garlic. The compound, i.e., allicin, is oily and unstable, and can be synthesized by mild oxidation of diallyl disulfide (174).

In late 1940s, Stoll and Seeback isolated, identified, and synthesized an oxygenated sulfur amino acid from garlic, i.e., alliin. They found that alliin was the parent compound of allicin and could be converted to allicin by the enzyme alliinase, which is released by crushing or mincing the garlic (175).

In the early 1960s, both Virtanen’s group in Finland and Suzuki’s group in Japan found that garlic contained \( \gamma \)-glutamylcysteines such as \( \gamma \)-glutamyl-S-allylcysteine (176, 177). \( \gamma \)-glutamyl-S-allylcysteine may be the parent compound of alliin (178).

Since these pioneering studies were conducted, a number of reports have been published regarding organosulfur compounds and their precursors in garlic and other Allium plants (179, 180).
1.3.2.2 Transformation of organosulfur compounds of garlic

It is well known that organosulfur compounds in garlic are transformed into a variety of compounds depending upon the processing method applied to raw garlic. Therefore, different processing procedures result in the generation of various kinds of chemical constitutes in garlic preparations (181).

One of the organosulfur components in intact garlic, the odorless amino acid, alliin (S-allylcysteine sulfoxide), is enzymatically converted into allicin when raw garlic cloves are crushed. As shown in Figure 1.4, allicin (diallyl thiosulfinate) is extremely unstable and readily converted into various compounds such as diallyl polysulfides, ajoene and vinyl dithiins depending upon the preparation methods (182). For example, allicin is mainly transformed into allyl sulfides in water or steam, and diallyl disulfide in alkaline condition (183-185). Allicin is converted to vinyl dithiins and ajoenes in organic solvents such as hexane, ether, vegetable oil and alcohol (181, 186).

γ-glutamylcysteines are another type of organosulfur compounds found in intact garlic. They are present only in the bulbs of the plants and only shortly before harvest (172). The γ-glutamylcysteines are very important as reserve compounds for producing additional alliin and isoalliin during wintering and sprouting, and they increase the antibiotic capacity of the young plants (187). They are nearly as abundant as the cysteine sulfoxides (alliin) on a weight basis. γ-glutamylcysteines can be gradually converted to S-alk(en)ylcysteines through an enzymatic process (Figure 1.5, 182) and may be further utilized to synthesize alliin or isoalliin (172).
Figure 1.4 Transformation of alliin of crushed raw garlic

All-: CH$_2$=CHCH$_2$-
**Figure 1.5** Transformations of γ-glutamylcysteines into S-alk(en)ylcysteines

Only trace amounts of S-alk(en)ylcysteines have been reported in raw garlic cloves. However, the amounts increase considerably when garlic cloves are dried in the manufacturing of commercial garlic powder and even more when chopped garlic is aged in water or dilute ethanol for three months or longer (172).

### 1.3.2.3 Major organosulfur compounds in various garlic products

Different garlic preparation methods result in many kinds of organosulfur compounds as indicated previously. Commonly available forms of garlic products are briefly described below and summarized in Table 1.1.

#### 1.3.2.3.1 Garlic powder

Garlic powders represent the composition of garlic cloves better than any other type of processed garlic because they are simply dehydrated, pulverized, peeled cloves. However, some changes do occur during processing depending on how the cloves are cut and dried.
The very best way to prepare a garlic powder with virtually no changes in composition involves freezing the garlic cloves in liquid nitrogen, followed by cold pulverization and vacuum drying. This procedure is time-consuming and very expensive. Most commercial garlic powders are prepared by cutting the peeled cloves into smaller pieces, then oven drying at 50-60°C. Neither alliinase nor alliin are affected at these temperatures (172, 188).

An appropriately processed garlic powder will contain an approximately 2.5-fold higher concentration of non-aqueous ingredients than fresh garlic. Small amounts of S-alk(en)ylcysteines such as S-allyl and S-methyl cysteines, and even smaller amounts of dialk(en)yl sulfides, can be detected in garlic powder due to processing (188).

1.3.2.3.2 Steam-distilled garlic oil

Steam-distilled garlic oil is frequently referred to as “essential oil of garlic”. However, this name is a misnomer because the compounds present in the oil of steam-distilled garlic are not present in whole or crushed garlic cloves but are produced from the thiosulfinates upon steam treatment of crushed garlic. The oil contains mainly allyl sulfides, especially di- and polysulfides such as diallyl disulfide and diallyl trisulfide. Allyl di- and polysulfide may comprise 86% of the total sulfides (172, 188, 189). Commercial steam-distilled garlic oil products diluted with a vegetable oil are by far the most common kind of garlic oil in the European and U.S. markets (188).

1.3.2.3.3 Oil-macerated garlic products

This type of processed garlic is much more common in Europe than in the United States. The production of oil-macerates is accomplished by grinding garlic cloves and mixing
the product with vegetable oils such as soybean oil and peanut oil, and then isolating the clear oil. Major sulfur compounds of this oil are vinyldithiins (70%), sulfides (18%) and ajoenes (12%). Vinyldithiins and ajoenes are the unique compounds in oil-macerated garlic products (172, 188).

1.3.2.3.4 Aged alcoholic garlic extract

This garlic product is prepared by incubating or aging chopped garlic in 15-20% ethanol for 18-20 months at ambient temperature. The incubation medium or extract is then filtered and concentrated to dryness and is sold in both dry and liquid forms (172). The main sulfur compounds in aged garlic extracts (AGE) are S-allyl cysteine, S-allylmercaptocysteine, S-1-propenylcysteine. The amount of S-allyl cysteine varies among preparations, ranging from 7.2 mg/g dry extract in some products to 0.37 mg/mL in others (172).

Table 1.1 Comparison of organosulfur compounds in various garlic products

<table>
<thead>
<tr>
<th>Products</th>
<th>Major organosulfur compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garlic Powder</td>
<td>γ-glutamyl cysteines, alliin, small amounts of S-alkylcysteines</td>
</tr>
<tr>
<td>Steam-distilled garlic oil</td>
<td>Allyl sulfides, especially di- and polysulfides</td>
</tr>
<tr>
<td>Oil-macerated garlic products</td>
<td>Vinyldithiins (70%), ajoenes (12%), sulfides (18%)</td>
</tr>
<tr>
<td>Aged alcoholic garlic extracts</td>
<td>S-alkylcysteines, mainly S-allyl cysteine, S-allylmercaptocysteine</td>
</tr>
</tbody>
</table>

1.3.3 Therapeutic actions of garlic and its preparations

Cardiovascular protective effects are reviewed in details in 1.3.4. Other therapeutic effects are briefly discussed below.
1.3.3.1 Antimicrobial effects

As early as in 1858, Louis Pasteur reported that garlic is antibacterial. Later Albert Schweizer is said to have made use of garlic in Africa for the treatment of amoebic dysentery (167). In the two world wars garlic was used as an antiseptic in the prevention of gangrene (167). Garlic was found to be effective against many gram-negative and gram-positive bacteria. The antimicrobial effects of garlic were extended to include 30 strains of mycobacteria consisting of 17 species and a number of medically important fungi (190). Garlic was used successfully to prevent and treat experimental infections with *Candida albicans* in chickens as well (191). A recent study reported that garlic oil, garlic powder and their diallyl constituents had anti-*Helicobacter pylori* activity (192). *Helicobacter pylori* is now believed to be the causal agent of chronic gastritis, and gastric and duodenal ulcers (192). Thus, garlic appears to have a broad-spectrum of antimicrobial activity.

The major active antimicrobial component of garlic is allicin. Another candidate may be ajoene (193). Removal of the thiosulfinates such as allicin by chloroform destroys the antibacterial and antifungal properties of aqueous garlic extracts (190).

1.3.3.2 Anticarcinogenic and antitumorigenic effect

Several epidemiological studies have shown that garlic consumption is correlated to reduced cancer risk. A study comparing populations of different regions of China reported that stomach cancer mortality rate was 13 times lower in individuals consuming about 20 g of garlic per day than in those who consumed less than 1 g per day (194, 195). Similar beneficial effects of garlic were observed in other countries including Italy and the United
States (196, 197). In the Iowa Women’s Health Study, the intake of 127 foods was determined in 41,387 women, followed by a five-year monitoring of colon cancer incidence. The results showed that garlic was the only food that had a statistically significant association with decreased colon cancer risk (196). The Italian study of 1,016 patients and 1,159 controls found that garlic, onion and vegetable decreased the risk of gastric cancer (197).

Animal and in vitro studies have demonstrated that various garlic constitutes inhibit the growth of different forms of cancers or cancer cells such as skin, mouth, mammary, lung, esophagus, stomach and colon tumors (198-203). Garlic powder, S-allyl cysteine (SAC), or diallyl disulfide (DADS) decreased the incidence of colon cancer and mammary tumor induced by chemical carcinogens in animals and reduced the number of tumors per animal (198-200). The studies have also found that allyl sulfides inhibit tumor cell proliferation in culture (201-203). The decreased proliferation is associated with a depression in cell cycle progression and the induction of apoptosis (204). SAC and S-propyl cysteine inhibited the in vitro formation of N-nitrosomorpholine (NMOR), a known carcinogen (205).

1.3.3.3 Other beneficial effects

Garlic has been shown to exhibit hypoglycemic activity and improve glucose intolerance in diabetic animals (206-208). Garlic or its constitutes can enhance immune functions such as augmenting macrophage and T-lymphocyte functions, and increasing interleukin production (209-210). Feeding aged garlic extract to Senescence-Accelerated Mice (SAM) demonstrated that the mice had a longer life span, improved learning, and reduced memory impairment (211, 212).
1.3.4 Cardiovascular protective effects

Extensive studies have demonstrated that garlic may reduce the risk of cardiovascular disease (213-215). In a randomized, double-blind, placebo-controlled clinical trial, garlic supplementation significantly reduced the increase in arteriosclerotic plaque volume by 5-18% or even effected a slight regression within the observational period of 48 months (216). Garlic could lower the risk of CVD through many ways including reducing plasma lipids, lowering blood pressure, depressing platelet adhesion and aggregation, and increasing fibrinolytic activity. Thus garlic provides a wide potential protection of the cardiovascular system (217).

1.3.4.1 Antithrombotic effect

Garlic extracts have been shown to inhibit platelet aggregation in vitro (218-220), and administration of garlic extracts to humans inhibits platelet aggregation ex vivo (221). It has been reported that AGE supplementation to humans (7.2 g/day for 4-6 months) significantly reduced epinephrine- and collagen-induced platelet aggregation, and decreased platelet adhesion to fibrinogen (217). Platelet adhesion and aggregation contribute to arterial thrombosis by promoting blood clotting, favoring vasoconstriction, activating the procoagulant capacity of endothelium and stimulating inflammation (222).

In addition, supplementation of aqueous extract of garlic (50 or 500 mg/kg) to animals suppressed serum or plasma thromboxane-B2 (TXB2) synthesis in vivo (223, 224). Serum TXB2 was reduced 80% in male subjects who consumed one clove (approximately 3 g) of fresh garlic daily for a period of 26 weeks (225). Raw garlic may be more potent
inhibitors of blood platelet aggregation and thromboxane synthesis than cooked or boiled garlic (220, 224).

Although the mechanism of antithrombotic effect of garlic is not clear, it may involve altering metabolism of arachidonic acid (219) or increasing production of nitric oxide by activation of nitric oxide synthase (226). Nitric oxide is an important inhibitor of platelet aggregation (227).

### 1.3.4.2 Effects on blood pressure

The hypotensive effects of garlic have been demonstrated in animals such as dogs, rabbits and rats (228-231), and in humans as well (232-234). Garlic powder decreased diastolic blood pressure from 102 mmHg to 91 mmHg after 8 week supplementation and to 89 mmHg after 12 week supplementation in mild hypertension patients (235). A meta-analysis revealed a decrease of 10 mmHg in systolic blood pressure and a decrease of 7 mmHg in diastolic pressure in garlic-treated subjects (236).

The hypotensive action of garlic may be due in part to a direct relaxant effect on smooth muscles (237) and may also involve nitric oxide production (238). Nitric oxide is a known relaxing factor (239). Hypertension is one of major risk factors for CHD (5).

### 1.3.4.3 Antioxidant effects

Garlic extracts reduce lipid peroxidation and free radicals in vitro system (240, 241). SAC and S-allylmercaptocystene (SAMC) have been shown to scavenge active oxygen species such as hydrogen peroxide (242, 243). Aged garlic extracts (AGE) inhibited Cu\(^{2+}\)-induced LDL oxidation in a concentration-dependent manner in vitro. SAC, SAMC, N-acetyl
SAC, and alliin decreased Cu\(^{2+}\)-induced LDL oxidation as well (244). Garlic extracts and its constitutives such as SAC protected endothelial cells from oxidized LDL-induced injury in vitro (245). In a randomized, double-blind, placebo-controlled crossover trial, after a two week supplementation of 600 mg daily of garlic powder to healthy subjects, the ex vivo susceptibility of apolipoprotein B-containing lipoproteins to oxidation was significantly decreased (-34\%) (246). A recent human study demonstrated that LDL isolated from subjects receiving 2.4 g AGE daily for a week was more resistant to oxidation than that from subjects receiving no supplement (247).

In addition, garlic supplementation has been demonstrated other cardioprotective effects such as increased fibrinolytic activity in animals and humans (248-251). Garlic may also have direct influence on the artery wall in preventing onset and development of atherosclerotic lesions and inducing regression of the lesions (252). Garlic inhibited proliferation of atherosclerotic cells and other cell types (253, 254), and reduced intracellular accumulation of free cholesterol, triglycerides and cholesterol esters (254, 255).

1.3.4.4 Cholesterol and lipid-lowering effects

Epidemiological studies on the cardiovascular protective effect of garlic are lacking. One early study in 1970s demonstrated that people who regularly ate larger amounts of garlic and onions had lower lipid and cholesterol levels than people who refrained from eating these vegetables (256), indicating that garlic and onions might have beneficial effects on the cardiovascular system by lowering blood lipid levels. This short term study was conducted on healthy subjects. It would be more convincing for a long term follow-up study to
determine the prevalence, morbidity, and mortality of cardiovascular disease in people who consume different amounts of garlic and onions.

Following this epidemiological study experiments have been conducted to investigate the lipid-lowering effects of garlic (232, 235, 257-269). Garlic supplementation has been clearly shown to decrease plasma cholesterol and/or triglyceride levels in animals such as chicken, pullets, rabbits, and rats (257-262).

In addition to animal experiments many human studies have demonstrated the lipid-lowering effects of garlic supplementation, especially on total cholesterol and LDL cholesterol levels (225, 232, 235, 263-269). Meta-analyses showed that plasma cholesterol concentration was reduced 9% to 12% in subjects treated with garlic as compared to a placebo group (270, 271). Garlic also reduces blood triglyceride levels (235, 272). In a recent clinical study, garlic supplementation reduced total serum cholesterol and triglycerides, and increased HDL-cholesterol in CAD patients (273). However, several recent human studies failed to confirm this effect of garlic (274-278). One possible reason for the discrepancy of lipid-lowering effect of garlic is the different garlic preparations used in the clinical trials such as garlic powder, garlic oil, and aged garlic extracts. Therefore, active constituents and/or the amounts of active constituents may not have been present or sufficient to produce the desired effect. Even with a single brand of garlic powder (Kwai), the results of clinical trials were markedly different. The earlier studies (235, 265-267) demonstrated significant reduction in serum cholesterol in the subjects in contrast to the negative results in recent studies (274, 276, 277). Lawson et al. (279) reported that the failure of recent clinical trials (274, 276, 277) with Kwai garlic powder might be due to the lower release of allicin in the newer lots (1993-1998) compared with the older lots manufactured before 1993. A different
brand garlic powder used by Gardner et al. (278) showed the lower release of allicin as well (279). In addition, garlic oil used by Berthold et al. (275) was found to have poor bioavailability (279). Hence, the subjects in the recent negative clinical trials probably received considerably less allicin than those in the older positive studies, which possibly accounted for much of the discrepancy in the outcomes. It is also noteworthy whether the garlic preparations can retain sufficient amounts of allicin to produce the claimed beneficial effects because allicin is a very labile compound (182). Another reason for the observed difference could be methodological differences in various clinical trials such as the subjects and the duration of supplementation. Table 1.2 lists the comparison of the clinical trials.

Although the mechanisms by which garlic lowers plasma lipids are not fully elucidated, garlic has been shown to decrease hepatic cholesterol and triglyceride synthesis in cultured hepatocytes (136, 280-283), which in turn may lead to a reduction of plasma cholesterol and triglycerides. In an in vitro study using liver homogenates, chloroform extracts of garlic, and five sulfur-containing compounds, i.e., ajoene, methylajoene, allicin, 2-vinyl-4H-1,3-dithiin, and diallyl disulfide were shown to inhibit cholesterol synthesis (284). Similarly, the mechanisms of inhibition on cholesterol and triglyceride biosynthesis are not clear. Animal studies have demonstrated that garlic-supplemented diets decrease the enzyme activities of various lipogenic enzymes such as FAS, G6PDH, and the cholesterogenic enzyme HMG-CoA reductase (257-259, 285, 286). Therefore, it is reasonable to speculate that the lipid-lowering effect of garlic may result from decreased lipogenesis and cholesterologenesis. In an early study with cultured rat hepatocytes by Yeh and Yeh (136) garlic extracts significantly inhibited [14C]acetate and [3H]glycerol incorporation into cholesterol and triglycerides, respectively, and the reduction of [3H]glycerol
incorporation into triglycerides was observed only in the presence of acetate, but not in the presence of oleate. It has been also shown that garlic supplementation decreased the biosynthesis of fatty acids from $[^{14}\text{C}]$acetate in rabbits (214). These findings suggest that the hypocholesterolemic effect of garlic may stem, in part, from decreased hepatic cholesterol synthesis, whereas the triglyceride-lowering effect appears to be due to inhibition of fatty acid synthesis. However, more studies are needed to confirm these findings and further investigate lipid-lowering effects of individual garlic compounds that are found in various garlic preparations.
Table 1.2 The comparison of study characteristics and results of clinical trials with garlic supplementation

<table>
<thead>
<tr>
<th>First Author (Year) (Ref.)</th>
<th>Experimental design</th>
<th>Garlic preparation and dosage</th>
<th>Subjects (N)</th>
<th>Duration of intervention</th>
<th>Total Cholesterol (mg/dl) Before</th>
<th>After</th>
<th>Triglyceride (mg/dl) Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bordia (1981) (263)</td>
<td>Randomized, placebo-controlled</td>
<td>Essential oil 0.25mg/kg BW/day</td>
<td>CHD patients (62)</td>
<td>10 months</td>
<td>T: 298</td>
<td>P: 280</td>
<td>228*</td>
<td>282</td>
</tr>
<tr>
<td>Lau (1987) (264)</td>
<td>Randomized, placebo-controlled</td>
<td>Kyolic aqueous extract 1000mg/day</td>
<td>Patients with hyperlipidemia (27)</td>
<td>24 weeks</td>
<td>T: 306</td>
<td>P: 302</td>
<td>262*</td>
<td>292</td>
</tr>
<tr>
<td>Auer (1990) (235)</td>
<td>Randomized, placebo-controlled</td>
<td>Garlic powder (Kwai) 600mg/day</td>
<td>Patients with mild hypertension (47, Hyperlipidemia (21))</td>
<td>12 weeks</td>
<td>T: 268</td>
<td>P: 267</td>
<td>230*</td>
<td>248</td>
</tr>
<tr>
<td>Mader (1990) (265)</td>
<td>Randomized, placebo-controlled</td>
<td>Garlic powder (Kwai) 800mg/day</td>
<td>Patients with hyperlipidemia (221)</td>
<td>16 weeks</td>
<td>T: 266</td>
<td>P: 262</td>
<td>235*</td>
<td>255</td>
</tr>
<tr>
<td>Vorberg (1990) (266)</td>
<td>Randomized, placebo-controlled</td>
<td>Garlic powder (Kwai) 900mg/day</td>
<td>Hypercholesterolemic patients (40)</td>
<td>16 weeks</td>
<td>T: 294</td>
<td>P: 288</td>
<td>232*</td>
<td>279</td>
</tr>
<tr>
<td>Jain (1993) (267)</td>
<td>Randomized, placebo-controlled</td>
<td>Garlic powder (Kwai) 900mg/day</td>
<td>Hyperlipidemic patients (42)</td>
<td>12 weeks</td>
<td>T: 262</td>
<td>P: 276</td>
<td>247*</td>
<td>274</td>
</tr>
<tr>
<td>Simon (1995) (274)</td>
<td>Randomized, placebo-controlled</td>
<td>Garlic powder (Kwai) 900mg/day</td>
<td>Hypercholesterolemic patients (28)</td>
<td>12 weeks</td>
<td>T: 258</td>
<td>P: 258</td>
<td>251</td>
<td>250</td>
</tr>
<tr>
<td>Steiner (1996) (232)</td>
<td>Randomized, placebo-controlled</td>
<td>Aged garlic extract 7.2g/day</td>
<td>hypercholesterolemic men (41)</td>
<td>6 months</td>
<td>6-7% reduction</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Yeh (1997) (269)</td>
<td>Randomized, placebo-controlled</td>
<td>Aged garlic extract 7.2g/day</td>
<td>hypercholesterolemic men (34)</td>
<td>5 months</td>
<td>T: 246</td>
<td>P: 243</td>
<td>228*</td>
<td>245</td>
</tr>
<tr>
<td>Berthold (1998) (275)</td>
<td>Randomized, placebo-controlled, crossover study</td>
<td>Garlic oil (steamed distilled) = oil from 4-5g fresh garlic/day</td>
<td>hypercholesterolemic men (25)</td>
<td>12 weeks</td>
<td>T: 291</td>
<td>P: 291</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bordia (1998) (273)</td>
<td>placebo-controlled</td>
<td>Garlic oil (from 4g raw garlic daily)</td>
<td>patients with coronary artery disease (60)</td>
<td>3 months</td>
<td>T: 253</td>
<td>P: 253</td>
<td>221*</td>
<td>249</td>
</tr>
<tr>
<td>Isaacsohn (1998) (276)</td>
<td>Randomized, placebo-controlled</td>
<td>Garlic powder (Kwai) 900mg/day</td>
<td>Hypercholesterolemic patients (50)</td>
<td>12 weeks</td>
<td>T: 274</td>
<td>P: 250</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Superko (2000) (277)</td>
<td>Randomized, placebo-controlled</td>
<td>Garlic powder (Kwai) 900mg/day</td>
<td>Hypercholesterolemic patients (50)</td>
<td>12 weeks</td>
<td>T: 250</td>
<td>P: 239</td>
<td>247</td>
<td>240</td>
</tr>
<tr>
<td>Gardner (2001) (278)</td>
<td>Randomized, placebo-controlled</td>
<td>Garlic powder, 500(H) and 1000 (F) mg/day</td>
<td>Hypercholesterolemic patients (51)</td>
<td>12 weeks</td>
<td>T: 231(H)</td>
<td>P: 235(F)</td>
<td>234</td>
<td>225</td>
</tr>
</tbody>
</table>

*Ref, reference; "N, numbers of subjects; 'T, treatment group; "P, placebo group; 'ND, no difference; *statistically different from placebo at p <0.05.
1.4 REFERENCES


12. The pooling project research group (1978) Relationship of blood pressure, serum cholesterol, smoking habit, relative weight and ECG abnormalities to incidence of major coronary events: final report of the pooling project. J. Chronic Dis. 31:201-306.


Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J. Nutr. 129:1135-1139.


CHAPTER 2

RESEARCH RATIONALE, OBJECTIVES, HYPOTHESES, AND EXPERIMENTAL MODEL SYSTEM
2.1 RATIONALE OF THE PROPOSED STUDIES

Coronary heart disease is the most important health problem and the leading cause of death in the United States (1). Increased blood levels of total cholesterol and its principal carrier, low density lipoprotein cholesterol are major risk factors for the development of coronary heart disease (CHD) (2). There is substantial evidence that lowering total and LDL-cholesterol levels can reduce the incidence of CHD and coronary death (2). Many primary and secondary prevention trials have proven the beneficial effect of lowering cholesterol (3-5). In addition to cholesterol, elevated levels of plasma triglyceride have been associated with an increased risk of cardiovascular disease (6-8). However, whether hypertriglyceridemia is an independent factor after controlling for other lipid risk factors, especially HDL-cholesterol level, was disputed until the reports published in 1998 (9, 10). The meta-analysis that employed the multivariate approach revealed that elevated triglycerides were independently associated with coronary artery disease incidence. In fact, increased triglyceride level as a risk factor for cardiovascular disease is independent of HDL cholesterol level (9, 10). The result suggests that triglyceride-rich lipoproteins such as IDL and VLDL are atherogenic. Studies in both human and animal models further showed that triglyceride-rich lipoproteins were related to the extent and severity of atherosclerosis (11). Conversely, the progression of coronary artery disease could be reduced with the reduction of triglyceride-rich lipoproteins (12).

Lifestyle changes are the foundation of the lipid-lowering therapy for reducing CHD risk (13). Lifestyle interventions include diet modification, physical activity and weight control. For diet modification, the National Cholesterol Education Program (NCEP) expert
panel recommended reduced intakes of saturated fats (< 7% of total calories) and cholesterol (< 200 mg per day) (13). PUFA and MUFA can decrease plasma lipids, and hence PUFA may be up to 10% of total calories and MUFA may be up to 20% of total calories. Total fat can be 25-35% of total calories. They also recommended increased intakes of other dietary constituents such as plant stanols/sterols and soluble fiber. However, in addition to stanols/sterols and soluble fiber, other dietary components such as soy protein and garlic compounds have been found to reduce blood lipid levels (14-16).

Garlic is commonly used as a food and condiment. Studies have found that garlic decreases plasma lipids, especially total cholesterol and LDL cholesterol, in both humans and animals (17-25). Garlic and other dietary constituents may complement or even potentiate the effects of the diet currently recommended by NCEP and AHA (26). The hypocholesterolemic effect of garlic was substantiated by meta-analyses showing that plasma cholesterol concentration was reduced 9% to 12% in subjects treated with garlic as compared to a placebo group (27, 28). Additionally, garlic has been shown to reduce blood triglyceride levels (17, 18, 29, 30). A more recent clinical study demonstrated that garlic supplementation significantly decreased total serum cholesterol and triglycerides and increased HDL-cholesterol in patients with coronary artery disease (31). In contrast to these reports, several recent human studies failed to confirm the cholesterol-lowering action of garlic (32-35). Although the reason for the difference is not readily known, factors such as experimental design and methodologies used in the clinical trials must be considered. Some examples of the factors are the duration of supplementation, subject’s nutrient intake, subject’s health condition and lipid profile. Another important reason for the observed discrepancy of lipid-lowering effect of garlic is the different garlic preparations used in the clinical trials such as
garlic powder, garlic oil, and aged garlic extracts. It has been shown that the organosulfur compounds are responsible for the lipid-lowering effects of garlic, and garlic preparations contain a wide variety of organosulfur compounds (36). Garlic powder contains γ-glutamyl cysteines, alliin, small amounts of S-alkylcysteines, lipid-soluble thiosulfinates such as allicin, thiosulfinate transformation products, and sulfides. Garlic oil mainly contains allyl sulfides. Aged garlic extract contains significant amount of water-soluble organosulfur compounds including S-alk(en)yl cysteines, S-allylmercaptocysteine, and γ-glutamyl-S-alk(en)yl cysteines (36). Thus, active constituents and/or the amounts of active constituents in the different garlic preparations may not be present or be sufficient to produce the lipid-lowering effect. However, the active ingredients responsible for the lipid-lowering effect of garlic are poorly understood. Therefore, further studies are needed to identify the active ingredients responsible for the lipid-lowering effect of garlic.

Although the mechanisms of garlic action are not fully known, garlic has been shown to decrease hepatic cholesterol and triglyceride synthesis (37-40), which in turn may lead to a reduction of plasma cholesterol and triglycerides. Furthermore, the mechanisms of inhibition of hepatic cholesterol and triglyceride biosyntheses are not clear. Several animal studies have suggested that garlic-supplemented diets decrease the enzyme activities of various lipogenic enzymes such as fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PDH), and the choleseterogenic enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (21, 22, 41, 42). Therefore, the lipid-lowering effect of garlic could result from impaired lipogenesis and cholesterolgenesis. The liver is the major site for the synthesis of endogenous lipids (43). The lipids are assembled into VLDL in liver and secreted into the blood (43). VLDL is the major carrier of triglyceride and LDL precursor after being hydrolyzed to IDL
in circulation, whereas LDL is the main carrier of cholesterol as described in Section 1.2.3. An early study with cultured rat hepatocytes by Yeh and Yeh (40) has demonstrated that garlic extracts significantly inhibited [14C]acetate and [3H]glycerol incorporation into cholesterol and triglycerides, respectively. Furthermore, the reduction of [3H]glycerol incorporation into triglycerides was observed only in the presence of acetate, but not in the presence of oleate (40). These findings suggest that the hypocholesterolemic effect of garlic results in part from decreased hepatic cholesterol synthesis, whereas the triglyceride-lowering effect appears to be due to inhibition of fatty acid synthesis. Therefore, the present studies were designed to identify the active compounds responsible for the lipid-lowering effects and the underlying mechanisms of this effect.

### 2.2 OBJECTIVES

The overall objective of the study was to identify the active compounds of garlic responsible for the lipid-lowering effect and to understand the mechanism(s) underlying the inhibitory effects on cholesterol and triglyceride syntheses in cultured rat hepatocytes. The specific aims were:

1) To determine the inhibitory potency of selected organosulfur compounds derived from garlic on cholesterol synthesis and the cytotoxicity of these compounds.

2) To evaluate the effects of water-soluble organosulfur compounds on triglyceride synthesis, fatty acid synthesis, and the activities of important lipogenic enzymes including fatty acid synthase and glucose-6-phosphate dehydrogenase.
3) To examine potential mechanisms by which S-alk(en)yl cysteines inhibit cholesterol synthesis. The effects of the compounds on HMG-CoA reductase were examined at transcriptional and post-transcriptional levels.

2.3 HYPOTHESES

There were four hypotheses tested in this study:

1) The potency of organosulfur compounds of garlic in inhibiting cholesterol synthesis varies depending on chemical structure and solubility of the compounds.

2) The reduction of triglyceride synthesis results from the inhibition of fatty acid synthesis. The decreased fatty acid synthesis is due to the depressed activity of lipogenic enzymes such as FAS and G6PDH.

3) The inhibition on cholesterol synthesis by organosulfur compounds of garlic stems in part from the depressed activity of HMG-CoA reductase. It is further hypothesized that the decreased activity of HMG-CoA reductase results from decreased gene expression and posttranslational modification.

2.4 EXPERIMENTAL MODEL SYSTEM

Liver plays the most important role in lipid homeostasis of the body including endogenous synthesis of lipids, production of VLDL, uptake of lipids from circulation, and the excretion of cholesterol from the body through bile as discussed in Section 1.2. Therefore, primary cultures of rat hepatocytes were used throughout this study. The main
reason for the use of cultured hepatocytes instead of liver in vivo is to minimize confounding factors associated with in vivo studies (44, 45). Hepatocyte cultures have proven applicable to a variety of experiments including studies of protein synthesis, metabolism of cholesterol and lipids, and especially screening of compounds for their metabolic activities (44, 46-48). The major advantage of such a system is that metabolic events can be studied in a defined, easily manipulated population of cells for periods of hours or days (45), i.e., the system permits evaluation of hepatocytes as pure isolates in a controlled environment (44). However, as an in vitro model system of hepatocyte culture, data derived from the studies may not be directly extrapolated to in vivo situation (49).

2.4.1 Optimal Conditions for Hepatocyte Culture

Hepatocytes were isolated from Sprague-Dawley male rats (200-300 g) by the method of Berry and Friend (50), as modified by Seglen (51), and detailed in chapter 3. The procedure involved collagenase perfusion via hepatic portal vein, tissue mincing, filtration and centrifugation. From each liver, 100-250 x 10^6 cells were obtained with a viability of 92-94% judging by trypan blue exclusion. The cells were resuspended in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units penicillin/mL, and 100 µg streptomycin/mL) and plated in each well of a six-well culture plate and incubated at 37°C under an atmosphere of 95% air and 5% CO₂. After 4 h of incubation, nonadhering cells were removed and discarded. Hepatocytes that adhered to the culture plate were refed with DMEM and incubated overnight for 16 h.
2.4.1.1 Cell adherence

A preliminary study was conducted to establish optimal cell adherence. Freshly isolated hepatocytes of different density were plated into culture wells. After 24 h incubation in DMEM, cells adhered to the culture plate were harvested and counted on hemocytometer under an inverted microscope. Percentage of cells adhered was calculated based on initial number of cells plated. The percent adherence of cell increased with increasing number of cells plated from $0.5 \times 10^6$ cells/well to $1.5 \times 10^6$ cells/well. Higher density of plated cells ($2.0 \times 10^6$ and $3.0 \times 10^6$ cells/well) did not further increase the percent adherence. The highest percent adherence was achieved by $1.0 \times 10^6$ and $1.5 \times 10^6$ cells/well (Figure 2.1). Therefore, the cell density of $1.0-1.5 \times 10^6$ cells/well was chosen and used throughout the study.

2.4.1.2 Correlation between cell number and cellular protein

Cells were plated in culture plates and the adhered cells were harvested as in 2.4.1.1. The harvested cells were counted on hemocytometer and the cellular protein was determined by the procedure of Lowry et al. (52). There was a strong linear relationship ($R^2=0.98$) between the cell number and cellular protein (Figure 2.2). Therefore, protein was measured for each experiment and used to determine the specific activity of enzyme activity and metabolic rates such as $[^{14}\text{C}]$acetate and/or $[^{14}\text{C}]$mevalonate incorporated into lipids.
Figure 2.1 Percent adherence of different cell densities. Data are expressed as means ± standard deviation of six determinations. Percent adherence is defined as the percent cell population adhered to the culture plate after 24 h incubation. Bars labeled with different letters are statistically different at \( p < 0.05 \).
Figure 2.2 The linear relationship between cell number and cellular protein. Cell number and protein values were obtained from the harvested cells after 24 h incubation. $R^2$ is the square of correlation coefficient.
2.4.2 Other methods used in the present studies

2.4.2.1 Metabolic study

After the overnight incubation, cells were washed with FBS-free DMEM and treated with test compounds at concentrations from 0.05 to 4 mmol/L in FBS-free DMEM with \(^{14}C\)acetate or \(^{14}C\)mevalonate as described in detail in chapter 3, 4, and 5. Four hours after incubation, the cells were harvested by scraping with a cell scraper for analyses. Cells isolated from same animals were used for \(^{14}C\)acetate incorporation into lipids and \(^{14}C\)mevalonate incorporation into cholesterol. Two or more experiments were carried to test the effects of each compounds.

2.4.2.2 Lipid analysis

The harvested cells were mixed with 20 mL of chloroform/methanol (2:1, vol/vol) and lipids were extracted according to the method of Folch et al. (53). The lipid extract was saponified with 6 mL of 3.75% methanolic KOH at 90 °C for 4 h (54). Cholesterol was isolated from non-saponifiable fraction of the lipid extract with digitonin precipitation (55) and described in chapter 3. Fatty acids were extracted from saponifiable fraction of the lipid extract and TG was isolated from the lipid extract by thin-layer chromatography (56) as described in chapter 4. The radioactivity of isolated cholesterol, fatty acids and TG was determined by liquid scintillation counting. The specific activity of cholesterol, fatty acids and TG synthesis was expressed as pmol acetate and/or mevalonate incorporated into corresponding lipids/mg cellular protein.
2.4.2.3 Determination of enzyme activities

Lactate dehydrogenase (LDH) activity was measured according to the method of Chao et al. (57) with modification as indicated in chapter 3. Lipogenic enzymes, i.e., fatty acid synthase (FAS) and glucose-6-phosphate dehydrogenase (G6PDH) were determined spectrophotometrically by the methods of Nepokroeff et al. (58) and Deutsch (59), respectively, as detailed in chapter 4. HMG-CoA reductase activity was measured by radiochemical assay using thin-layer chromatography (TLC) (60) and described in chapter 5.

2.4.2.4 Measurement of HMG-CoA reductase mRNA and protein

The abundance of HMG-CoA reductase mRNA was quantified by real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (61) and discussed in detail in chapter 5. The amount of HMG-CoA reductase protein was determined by immunoblot analysis (62) and described in chapter 5.

2.4.2.5 Data analysis and statistics

Data are presented as means ± standard error of the mean (SEM) for samples combined from experiments for each compounds. The same population of cells isolated from one rat was used in each experiment. For $[^{14}C]$acetate incorporation into lipids and LDH release into the medium, eight samples from two experiments were combined. For $[^{14}C]$mevalonate incorporation into cholesterol, ten samples from three experiments were combined. Six samples from three experiments were combined for FAS, G6PDH and HMG-CoA reductase analyses. Five samples from two experiments were combined for HMG-CoA reductase mRNA analysis. Four samples from two experiments were combined for the
measurement of expressed and total activity of HMG-CoA reductase and the activity of the enzyme in lower dithiothreitol concentration. The comparisons of the test compounds were analyzed by analysis of variance (ANOVA). When statistical significance was indicated by ANOVA, the Fisher's multiple test and Dunnett test were applied to identify significant difference between the groups and the difference between the treatment and control, respectively, at \( p < 0.05 \).

2.4.3 Organosulfur compounds tested in the studies

Eleven water-soluble and six lipid-soluble compounds were tested in the studies. Water-soluble compounds included four S-alk(en)yl cysteines, i.e., S-allyl cysteine (SAC), S-ethyl cysteine (SEC), S-methyl cysteine (SMC), and S-propyl cysteines (SPC); three \( \gamma \)-glutamyl S-alk(en)yl cysteines, i.e., \( \gamma \)-glutamyl-S-allyl cysteine (GSAC), \( \gamma \)-glutamyl-S-methyl cysteine (GSMC), and \( \gamma \)-glutamyl-S-propyl cysteine (GSPC); S-allylmercaptocysteine (SAMC), S-allyl-N-acetyl cysteine (SANC), S-allylsulfonyl analine (SASA), and S-allyl cysteine sulfoxide (Alliin). Lipid-soluble compounds tested were: three diallyl compounds, i.e., diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS); two dipropyl compounds, i.e., dipropyl sulfide (DPS) and dipropyl disulfide (DPDS); and methyl allyl sulfide (MAS). The structures of the test compounds were illustrated in Figure 2.3.
Figure 2.3 Structures of test compounds in the studies
2.5 REFERENCES


CHAPTER 3

INHIBITION OF CHOLESTEROL BIOSYNTHESIS BY ORGANOSULFUR COMPOUNDS DERIVED FROM GARLIC

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3.1 ABSTRACT

The study was undertaken to test the inhibitory potential on cholesterogenesis of organosulfur compounds derived from garlic. The primary rat hepatocytes maintained in Dulbecco’s modified Eagle medium were treated with [2-14C]acetate as substrate for cholesterol synthesis in the presence or absence of test compounds at 0.05 to 4.0 mmol/L. Eleven water-soluble and six lipid-soluble compounds of garlic were tested. Among water-soluble compounds, S-allyl-cysteine (SAC), S-ethyl cysteine (SEC), and S-propyl cysteine (SPC) inhibited [2-14C]acetate incorporation into cholesterol in a concentration-dependent manner achieving 42% to 55% maximal inhibition. γ-Glutamyl-S-allyl cysteine, γ-glutamyl-S-methyl cysteine, and γ-glutamyl-S-propyl cysteine were less potent exerting only 16% to 29% maximal inhibitions. Alliin, S-allyl-N-acetyl cysteine, S-allylsulfonyl alanine, and S-methyl cysteine had no effect on cholesterol synthesis. Of the lipid-soluble compounds, diallyl disulfide (DADS), diallyl trisulfide (DATS), and dipropyl disulfide (DPDS) depressed cholesterol synthesis by 10% to 25% at low concentrations (≤ 0.5 mmol/L), and abolished the synthesis at high concentrations (≥ 1.0 mmol/L). Diallyl sulfide, dipropyl sulfide and methyl allyl sulfide slightly inhibited [2-14C]acetate incorporation into cholesterol only at high concentrations. The complete depression of cholesterol synthesis by DADS, DATS, and DPDS was associated with cytotoxicity as indicated by marked increase in cellular LDH release. There was no apparent increase in LDH secretion by water-soluble compounds except S-allyl mercaptocysteine, which also abolished cholesterol synthesis. Judging from maximal inhibition and IC50 (concentration required for 50% of maximal inhibition), SAC, SEC, and SPC are equally potent in inhibiting cholesterol synthesis.
3.2 INTRODUCTION

Garlic has been recognized for its medicinal potentials since ancient times, but only recently has evidence emerged that garlic may decrease hypercholesterolemia (1, 2) and reduce cancer risk (3). Extensive studies have been conducted to test anticarcinogenic and antitumorigenic properties of garlic and garlic components (4-9). It has been shown that S-allyl cysteine (SAC) and S-propyl cysteine (SPC) effectively blocked N-nitrosomorpholine (NMOR, a liver carcinogen) formation, and SAC and diallyl disulfide (DADS) reduced NMOR mutagenicity (10). Diallyl sulfides such as diallyl sulfide (DAS), DADS and diallyl trisulfide (DATS) have also been found to prevent benzo(a)pyrene-induced cancer in mice (11). To the contrary, less is known about garlic ingredients responsible for reducing plasma level of cholesterol.

Studies have shown garlic can decrease plasma lipids, especially total cholesterol and LDL cholesterol in humans and animals (1, 2, 12-17). The hypocholesterolemic effects were confirmed by meta-analyses showing a reduction of plasma cholesterol concentration between 9 and 12 % in subjects treated with garlic as compared to a placebo group (18, 19). A more recent clinical study reported that garlic supplementation significantly reduced total serum cholesterol and triglycerides, and increased HDL-cholesterol in patients with coronary artery disease (20). However, the most recent studies of Berthold et al. (21), Isaacsohn et al. (22) and Simons et al. (23) failed to confirm such a beneficial effect of garlic. Despite the discrepancy in these studies, garlic has been shown to decrease hepatic cholesterol synthesis (24-27), which may explain in part the hypocholesterolemic effect of garlic in humans and animals (1, 14, 16, 20). We recently have shown that water extract of garlic and water-soluble
component of garlic, SAC, depressed cholesterol synthesis in rat hepatocyte culture (27). Similarly, lipid-soluble sulfur compounds of garlic such as DADS, allicin and its derivative (ajoene) have been found to be potent inhibitors of cholesterol synthesis (26, 28). However, since a wide variety of organosulfur compounds has been identified and isolated from different garlic preparations (29, 30), it is essential to characterize the active ingredient(s) responsible for the cholesterol-lowering effect of garlic (30).

The present study was undertaken to identify the active compounds and their inhibitory potency of cholesterol biosynthesis in cultured rat hepatocytes. Additionally, the cytotoxicity of these compounds was determined and correlated to the cholesterol-lowering effect of the compounds.

3.3 MATERIALS AND METHOD

3.3.1 Chemicals

[2-14C] Acetate was purchased from Amersham Corp. (Arlington Heights, IL). Collagenase D was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Culture media, fetal bovine serum (FBS), penicillin and streptomycin were the products of GIBCO (Gaithersburg, MD). Organosulfur compounds tested in this study included: eleven water-soluble compounds; SAC, S-ethyl cysteine (SEC), S-methyl cysteine (SMC), SPC, γ-glutamyl-S-allyl cysteine (GSAC), γ-glutamyl-S-methyl cysteine (GSMC), γ-glutamyl-S-propyl cysteine (GSPC), S-allyl cysteine sulfoxide (i.e., Alliin), S-allyl-N-acetyl cysteine (SANC), S-allyl-mercaptocysteine (SAMC), and S-allylsulfonyl alanine (SASA); and six lipid-soluble compounds: DAS, DADS, DATS, dipropyl sulfide (DPS), dipropyl disulfide
(DPDS), and methyl allyl sulfide (MAS). All the water-soluble compounds were provided by Wakunaga of America Co., Ltd. (Mission Viejo, CA). Of the six lipid-soluble compounds tested, DAS, DADS, DPS, and DPDS were purchased from Fluka Chemika (Ronkonkoma, NY). MAS was obtained from Aldrich Chemical Co. (Milwaukee, WI). DATS was a generous gift from Dr. Milner’s laboratory (Pennsylvania State University, PA). All other chemicals of reagent grade were purchased from Sigma Chemical Co. (St. Louis, MO).

3.3.2 Animals

Male Sprague-Dawley rats (200-300 g) were obtained from Harlan Sprague-Dawley Co. (Indianapolis, IN) and fed a nonpurified diet (Purina Rat Chow, Ralston Purina, St. Louis, MO). The animals were housed individually in stainless steel cages at approximately 24°C and 50% relative humidity on a 12-h light/dark cycle (0600-1800). The animal protocol was approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

3.3.3 Hepatocyte culture

Liver cells were isolated from rats according to the method of Berry and Friend (31), as modified by Seglen (32). Briefly, rats were anesthetized with Nembutal (5 mg/100 g body weight), and the hepatic portal vein was cannulated for perfusion with buffer [NaCl, 142 mmol/L; KCl, 6.7 mmol/L; N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES), 10 mmol/L; NaOH, 5.5mmol/L; pH 7.4] for 15 min. Immediately after perfusion in situ, the liver was carefully excised and perfused with collagenase buffer (NaCl, 67 mmol/L; KCl, 6.7 mmol/L; HEPES, 100 mmol/L; CaCl₂·H₂O, 5.4 mmol/L; NaOH, 66 mmol/L; pH
7.6; 50 mg collagenase D/100 mL) for 10-15 min. The enzyme-treated liver was then subjected sequentially to mincing, incubation, filtration and centrifugation for cell isolation and purification. From each liver, 100-250 x 10⁶ cells were obtained with a viability of 92-94% as judged by trypan blue exclusion. The cells were resuspended in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS and antibiotics (100 units penicillin/mL, and 100 µg streptomycin/mL) to obtain 0.5-0.8 x 10⁶ cells/mL of suspension. Two-milliliter aliquots of the suspension were plated per well in a six-well culture plate (Becton Dickinson Labware, Lincoln, NJ) and incubated at 37 °C under an atmosphere of 95% air and 5% CO₂. After 4 h of incubation, nonadhering cells were removed and discarded. Hepatocytes that adhered to the culture plate were refed with DMEM and incubated for 16 h.

3.3.4 Metabolic studies

At the end of 20 h incubation, cells were washed three times with 2 mL of FBS-free DMEM, followed by incubation with 2 mL of the same medium containing sodium salt of [2-¹⁴C]acetate (specific activity, 37 MBq/mmol) and 0.5 mmol/L nonlabeled sodium acetate in the presence or absence of organosulfur compounds. The lipid-soluble organosulfur compounds were dissolved in dimethyl sulfoxide (DMSO) and then in DMEM. The final concentrations of DMSO did not exceed 2%, and appropriate controls containing DMSO were run. A preliminary study has established a linear rate of [2-¹⁴C]acetate incorporation into cholesterol between 4- and 12-hour incubation of hepatocytes. Thus, throughout the study cells were incubated for 4 hours. After the incubation, the medium was collected and cells were harvested with 1.3 mL of ice-cold water by scraping with a cell scraper.
3.3.5 Lipid analysis

The harvested cells were mixed with 20 mL of chloroform/methanol (2:1, vol/vol) to extract lipids according to the method of Folch et al. (33). For measurement of [2-14C]acetate incorporation into cholesterol, the lipid extract was saponified in 6 mL of 3.75% methanolic KOH at 90 °C for 4 h. Non-saponifiable lipids were extracted with petroleum ether (b.p. 35-60 °C), and cholesterol was precipitated with digitonin (34). Briefly, nonsaponifiable lipid fraction was evaporated to dryness under nitrogen stream and dissolved in acetone:ethanol (1:1, vol/vol). After the addition of 1% digitonin solution (in 50% ethanol and 1% acetic acid) the sample was allowed to stand overnight for digitonide formation. For further purification, digitonide was washed with acetone:ethyl ether (1:1, vol/vol) followed by final wash with ethyl ether alone. The radioactivity of 14C-labeled sterol digitonide was taken as a measure of cholesterol derived from [2-14C]acetate. The radioactivity was determined by liquid scintillation spectrometry (Beckman Model LS 3801; Beckman Instruments, Fullerton, CA). The specific activity of cholesterol synthesis was expressed as pmol acetate incorporated into cholesterol/mg cellular protein. Cellular protein was determined by the procedure of Lowry et al. (35). The relative rate of cholesterol synthesis by cells treated with organosulfur compounds was expressed as percentage of control calculated by specific activity of treatment group/specific activity of control non-treatment group x 100. IC50 (concentration required for 50% of maximal inhibition) were calculated by regression and correlation analysis between substrate concentration and inhibition percentage.
3.3.6 Determination of cytotoxicity

Cytotoxicity of hepatocytes was determined by measuring release of cellular lactate dehydrogenase (LDH) into the culture medium. The percentage of LDH released was estimated by dividing the activity in the medium by the sum of LDH activity in cells and medium x 100. Hepatocytes were lysed with 0.2% Triton X-100. LDH activity was measured according to the method of Chao et al. (36) with modification as follow: the reaction mixture contained 2.55 mL of potassium phosphate buffer (0.1 mol/L, pH 7.4) and 0.1mL of NADH (4.5 mmol/L). The cell lysate (0.05mL) was added and mixed. At time zero, the reaction was initiated by adding 0.1 mL of 20 mmol/L sodium pyruvate solution. The rate of decreasing absorbance at 340 nm was monitored at 25°C with a Beckman DU®-50 LS 5801 spectrophotometer (Beckman Instruments). The specific activity of the enzyme measured in hepatocytes of untreated group ranged from 1 to 2 µmol of substrate used /min/well.

3.3.7 Statistics

Data are presented as means ± standard error of the mean (SEM). The comparisons of the test compounds were analyzed by analysis of variance (ANOVA). When statistical significance was indicated by ANOVA, Fisher’s multiple test was applied to identify the significant difference between the groups at $p <0.05$.

3.4 RESULTS

Incorporation of [2-14C]acetate into cholesterol was measured in hepatocytes treated with or without organosulfur compounds at various concentrations (0.05-4.0 mmol/L)
throughout the study. As shown in Figure 3.1, the rate of [2-14C] acetate incorporation into cholesterol in the untreated group was arbitrarily defined as 100%. The rates of incorporation expressed as pmol acetate/mg cellular protein/4 h for the control varied from one experiment to another, but ranged from 868 to 1396 pmol acetate/mg cellular protein. Among S-alk(en)yl cysteines, SAC, SEC and SPC but not SMC inhibited [2-14C] acetate incorporation into cholesterol in a concentration-dependent manner with a maximal inhibition of 42-55%. The inhibition was apparent at a concentration as low as 0.05 mmol/L for SEC and SPC, and 0.1 mmol/L for SAC. All γ-glutamyl S-alk(en)yl cysteines (i.e., GSAC, GSMC and GSPC) inhibited the rate of cholesterol synthesis to a maximum of 16-30% (Figure 3.2). The inhibition was not seen at concentrations lower than 2.0 mmol/L for GSAC and GSPC, while a significant inhibition was noted at 0.5 mmol/L for GSMC. SAMC, a disulfur containing S-allyl cysteine derivative, inhibited the [2-14C] acetate incorporation into cholesterol by 7-17% at 0.05 and 0.5 mmol/L and diminished the incorporation into cholesterol at 2.0 and 4.0 mmol/L (Figure 3.3). Other water-soluble compounds (i.e., alliin, SANC and SASA) at concentrations from 0.05 to 4.0 mmol/L did not alter the rate of [2-14C] acetate incorporation into cholesterol (data not shown).

The inhibition of cholesterol synthesis by lipid-soluble compounds was determined in subsequent experiments. Of three sulfur containing diallyl compounds, DAS (monosulfide) exhibited the least effect with a maximum inhibition of 40% observed at 4.0 mmol/L concentration (Figure 3.4A). The rate of cholesterol synthesis was decreased by 25% by both DADS and DATS (polysulfides) at 0.05 mmol/L (Figure 3.4B and C). The synthesis was completely diminished by DATS and DADS at 1.0 and 2.0 mmol/L, respectively. The inhibition pattern by sulfur containing dipropyl compounds resembled that of DAS and
DADS. A maximum of 17% inhibition was achieved by DPS at 4.0 mmol/L (data not shown), while the synthesis was abolished by DPDS at the same concentration (Figure 3.4D). An inhibition (by 15%) was apparent beginning at 0.5 mmol/L for DPDS. The synthesis was inhibited 15% maximally by MAS, a sulfide containing two different alk(en)yl groups, i.e., methyl and allyl moieties (data not shown).

In order to determine whether the inhibitory effect of sulfur compounds on cholesterogenesis could also be attributed to cytotoxicity, cellular release of lactate dehydrogenase (LDH) into the medium was measured. Increased release of lactate dehydrogenase by hepatocytes into culture medium is widely used as an index of cytotoxicity (36, 37). For hepatocytes treated without water-soluble compounds, the amount of LDH activity recovered in the medium accounted for 13-16% of total cellular LDH activity (Table 3.1). The treatment of cells with S-alk(en)yl cysteines and γ-glutamyl S-alk(en)yl cysteines except GSPC did not increase LDH release at 4.0 mmol/L (Table 3.1). However, GSPC did not alter percentage of LDH release at 2.0 mmol/L (data not shown). Unlike these findings, SAMC increased LDH release in a concentration dependent fashion and reached 70-78% at higher concentrations (2.0 and 4.0 mmol/L) (Figure 3.3).
Figure 3.1 Inhibition of $[2^{-14}\text{C}]$acetate incorporation into cholesterol by S-allyl cysteine (SAC), S-ethyl cysteine (SEC), S-methyl cysteine (SMC), and S-propyl cysteine (SPC) in primary hepatocyte culture. Data are expressed as percentage of the control and represent means ± SEM of eight samples. *Statistically different from controls, $p<0.05$. 
Figure 3.2 Inhibition of [2-14C]acetate incorporation into cholesterol by γ-glutamyl-S-allyl cysteine (GSAC), γ-glutamyl-S-methyl cysteine (GSMC), and γ-glutamyl-S-propyl cysteine (GSPC) in primary hepatocyte culture. Data are expressed as percentage of the control and represent means ± SEM of eight samples. *Statistically different from controls, $p<0.05$. 
Figure 3.3 Inhibition of $[^{2-14}C]$acetate incorporation into cholesterol and lactate dehydrogenase (LDH) release into the medium by S-allyl mercaptocysteine (SAMC) in primary hepatocyte culture. Data are expressed as a percentage of the control for $[^{2-14}C]$acetate incorporation into cholesterol (●) and a percentage of LDH activity in medium relative to total activity in cells and medium (○). The data represent means ± SEM of eight samples. *Statistically different from controls, $p < 0.05$. 
Figure 3.4 Inhibition of [2-14C]acetate incorporation into cholesterol and lactate dehydrogenase (LDH) release into the medium by diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), and dipropyl disulfide (DPDS) in primary hepatocyte culture. Data are expressed as a percentage of the control for [2-14C]acetate incorporation into cholesterol (●) and a percentage of LDH activity in medium relative to total activity in cells and medium (○), and represent means ± SEM of eight samples. *Statistically different from controls, p < 0.05.
Since lipid-soluble compounds were dissolved in DMSO to a final concentration of less than 2%, cells in the control group were incubated in the presence of DMSO at the same concentration. LDH release into the medium of the control ranging from 21-24% was higher than that observed in the cells not treated with DMSO (Figure 3.4 and Table 3.1). Incubation of cells with DAS and DPDS significantly increased percentage release of LDH at 0.5 mmol/L (Figure 3.4A and D). The increase was noted for DATS at 0.05 mmol/L and for DADS at 0.1 mmol/L (Figure 3.4B and C). Increasing concentration to 1.0 mmol/L of DATS, 2.0 mmol/L of DADS and 2.0 mmol/L of DPDS further increased LDH release to approximately 90%. At 4.0 mmol/L, DADS, DATS and DPDS did not further increase LDH release. DAS also elevated LDH release but only up to 50%. DPS, another monosulfur compound, increased LDH release to 33% only at 4.0 mmol/L, whereas MAS had no effect on the release (data not shown).

### Table 3.1 Release of cellular LDH into medium of water-soluble compounds in primary hepatocyte culture

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mmol/L)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>SAC</td>
<td>13.39 ± 0.94</td>
<td>14.50 ± 0.36</td>
</tr>
<tr>
<td>SEC</td>
<td>14.71 ± 1.08</td>
<td>15.43 ± 0.31</td>
</tr>
<tr>
<td>SPC</td>
<td>14.38 ± 1.96</td>
<td>14.09 ± 0.67</td>
</tr>
<tr>
<td>GSAC</td>
<td>14.72 ± 0.73</td>
<td>15.96 ± 0.91</td>
</tr>
<tr>
<td>GSMC</td>
<td>14.72 ± 0.73</td>
<td>14.96 ± 0.72</td>
</tr>
<tr>
<td>GSPC</td>
<td>15.82 ± 0.31</td>
<td>17.72 ± 1.03*</td>
</tr>
</tbody>
</table>

*aData are expressed as percentage of activity in medium relative to total activity in cells and medium, and represent means ± SD of eight samples. *Statistically different from controls, p<0.05. SAC, S-allyl cysteine; SEC, S-ethyl cysteine; SPC, S-propyl cysteine; GSAC, γ-glutamyl-S-allyl cysteine; GSMC, γ-glutamyl-S-methyl cysteine; GSPC, γ-glutamyl-S-propyl cysteine; LDH, lactate dehydrogenase.
Table 3.2 shows maximal inhibition and IC$_{50}$ for water-soluble compounds that exhibited concentration-dependent inhibition on cholesterol synthesis. The maximal inhibition was the highest for SPC followed in increasing order by SAC > SEC > GSMC > GSPC > GSAC, while the calculated IC$_{50}$ was the lowest for SEC followed in decreasing order by SAC < SPC < GSMC < GSAC < GSPC.

Table 3.2 Maximal Inhibition and Calculated IC$_{50}$ of Water-soluble Compounds on Cholesterol Synthesis$^a$

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (mmol/L)</th>
<th>Maximal inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAC</td>
<td>0.61</td>
<td>50</td>
</tr>
<tr>
<td>SEC</td>
<td>0.58</td>
<td>42</td>
</tr>
<tr>
<td>SPC</td>
<td>0.72</td>
<td>55</td>
</tr>
<tr>
<td>GSAC</td>
<td>1.66</td>
<td>16</td>
</tr>
<tr>
<td>GSMC</td>
<td>1.12</td>
<td>29</td>
</tr>
<tr>
<td>GSPC</td>
<td>1.92</td>
<td>18</td>
</tr>
</tbody>
</table>

$^a$IC$_{50}$, concentration required for 50% of maximal inhibition. SAC, S-allyl cysteine; SEC, S-ethyl cysteine; SPC, S-propyl cysteine; GSAC, $\gamma$-glutamyl-S-allyl cysteine; GSMC, $\gamma$-glutamyl-S-methyl cysteine; GSPC, $\gamma$-glutamyl-S-propyl cysteine.
3.5 DISCUSSION

Previous studies from our laboratory (27) and that of Gebhardt et al. (26, 28) have reported inhibitory effects of some garlic constituents on cholesterol biosynthesis in vitro. The present study not only confirmed the earlier findings but also expanded to test 11 water-soluble and 6 lipid-soluble compounds derived from garlic. When incubated with hepatocytes in culture, most water-soluble compounds were effective in decreasing [2-14C] acetate incorporation into cholesterol. All S-alk(en)yl cysteines except SMC inhibited cholesterol synthesis to various degrees. The differences among the S-alk(en)yl cysteines are the number of carbons and/or the number of double bonds. The potency of inhibition on cholesterol synthesis increased with increasing carbon number. SMC containing only one carbon (CH3-) in the alk(en)yl group, was ineffective while SEC containing two carbons (CH3CH2-) inhibited the rate of [2-14C] acetate incorporation into cholesterol. A greater inhibition was noted by SAC and SPC, both containing three carbons, but with a double bond (CH2=CHCH2-) in SAC and none (CH3CH2 CH2-) in SPC. Glutamylated products of S-alk(en)yl cysteines, i.e., GSAC, GSMC, and GSPC, were less potent than SAC, SEC, and SPC in inhibiting cholesterogenesis. However, unlike SMC, GSMC reduced [2-14C] acetate incorporation into cholesterol and was more effective than GSAC and GSPC. Interestingly, SAMC which contains one more sulfur atom than SAC was more potent than the latter in inhibiting [2-14C] acetate incorporation into cholesterol.

All six lipid-soluble compounds except MAS appeared to exert greater inhibition on [2-14C] acetate incorporation into cholesterol than water-soluble compounds. The degree of inhibition was associated with the number of sulfur atoms in the molecules. At
concentrations of 1.0 mmol/L or higher, the percentage of inhibition was in order of DATS > DADS > DAS. This is consistent with inhibition on the growth of canine mammary tumor cells in culture (8). Similarly, DPDS, a polysulfide compound, exerted a greater inhibition than a single sulfur containing DPS. However, it should be stressed that the marked decrease of [2-\(^{14}\)C] acetate incorporation into cholesterol by the lipid-soluble compounds was closely associated with the extensive cytotoxicity as indicated by markedly increased release of cellular LDH into the medium.

For determination of cytotoxicity, LDH released into culture medium was measured in cells treated with the test compounds. In the presence of water-soluble compounds, percentage release of LDH remained unchanged from that seen in control group (i.e., 13-16%). It should be noted that the control values were consistent with the range observed by other investigators (37-40), although values as low as 10 % and as high as 30 % have been reported (41). DMSO added to the incubation medium alone at a final concentration of 2% or less augmented LDH release to 21-24%. Nonetheless, in the presence of DMSO, most lipid-soluble compounds further increased LDH release in a concentration-dependent manner, especially for those containing at least two sulfur atoms, e.g., DADS, DATS and DPDS. Overall, the degree of LDH release was the greatest with DATS, followed in order by DADS, DPDS and DAS. The results therefore revealed that the more sulfur atoms in the molecule, the higher the cytotoxicity of the compounds. Consistent with this notion, SAMC, the only water-soluble compound with two sulfur atoms in the molecule, was also highly cytotoxic.

It should be pointed out that an earlier study by Gebhardt and Beck (28) showed that treatment of hepatocytes with DADS even at 10 mmol/L did not increase LDH release. The reason for the discrepancy observed between the studies is not known. However, it is worth
noting that in the latter study (28), the cells were pre-treated with the compound for 2 h in the culture medium. The medium was then replaced by new medium containing labeled acetate and incubated again for measurements of cholesterol synthesis and LDH release (28). In the present study, sulfur compounds were added directly to the culture medium and LDH release measured 4 h after incubation (27). Whether these different experimental conditions account for the observed difference warrants further investigation. At this juncture, it is interesting to note that the study of Abdul-Hussain and Mehandale (41) demonstrated that the degree of LDH leak through hepatocyte membrane was dependent on the duration of cell incubation in the medium (41).

The inverse relationship between cholesterol synthesis and LDH release let us to conclude the inhibition on cholesterogenesis by lipid-soluble compounds at concentration higher than 0.5 mmol/L was likely due to cytotoxicity rather than impairment on metabolic pathway of cholesterol synthesis. Similarly, the marked inhibition by SAMC was associated with cytotoxic effect. The unaltered percentage LDH release associated with most water-soluble compounds therefore strongly suggests that inhibition of cholesterol synthesis by these compounds likely results from impairment of specific enzyme(s) of cholesterogenic pathway.

A comparison of cytotoxicity and cholesterol inhibition potency of various compounds suggests that water-soluble compounds of garlic may be of more potential than lipid-soluble compounds in lowering plasma concentration of cholesterol. In fact in a recent clinical trial (21), garlic oil rich in polysulfides (42) given to patients with moderate hypercholesterolemia was ineffective in lowering serum cholesterol. On the other hand, aged garlic extract consisting mostly of water-soluble sulfur compounds supplemented to the diet
of hypercholesterolemic patients decreased plasma concentrations of total cholesterol and LDL-cholesterol 6-9% (1, 2). It is apparent from the present study that three S-alk(en)yl cysteines (i.e., SAC, SPC and SEC) effectively depressed hepatic cholesterogenesis at a low concentration and without a sign of cytotoxicity. However, SAC may be the most important contributor to the hypocholesterolic effect of aged garlic extract (1, 2) because SAC is a major sulfur-containing amino acid derivative in garlic, especially in aged garlic extract (43). Aged garlic extract is known to contain 456 µg SAC/g of dry powder (44).

The mechanisms underlying the inhibitory action of garlic compounds on cholesterol synthesis have not been fully elucidated. However, animal studies have suggested that garlic-supplemented diets decreased the cholesterogenic enzyme, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (16, 45). Sulfur compounds of garlic such as allicin have been shown to inhibit the activity of acetyl-CoA synthetase (46). Since [2-14C] acetate was used as a substrate in the present study, the activation of acetate to acetyl-CoA by the synthetase must also play a role in its incorporation into cholesterol.

In conclusion, although lipid-soluble compounds isolated from garlic appeared to abolish cholesterol synthesis, the inhibition may be due to cytotoxicity. Water-soluble components of garlic especially SAC appears to be most responsible for the reduction of cholesterol synthesis. Furthermore, the results suggest that the hypocholesterolemic effect of garlic results in part from impaired cholesterol synthesis.
3.6 REFERENCES


CHAPTER 4

WATER-SOLUBLE ORGANOSULFUR COMPOUNDS OF GARLIC INHIBIT
FATTY ACID AND TRIGLYCERIDE SYNTHESSES IN CULTURED RAT
HEPATOCYTES

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Permission was granted by Lipids on June 14, 2001 to include this chapter as part of the thesis.
4.1 ABSTRACT

The putative hypolipidemic effect of garlic remains controversial. To gain further insight into the effect of garlic on lipid metabolism, the present study determined the inhibitory effects of water-soluble organosulfur compounds present in garlic on triglyceride (TG) and fatty acid synthesis in cultured rat hepatocytes. When incubated at 0.05 to 4.0 mmol/L with cultured hepatocytes, S-allyl cysteine (SAC) and S-propyl cysteine (SPC) decreased [2-14C] acetate incorporation into triglyceride in a concentration dependent fashion achieving a maximal inhibition at 4.0 mmol/L of 43% and 51%, respectively. The rate of [2-14C] acetate incorporation into phospholipids was depressed to a similar extent by SAC and SPC. SAC, SEC, γ-glutamyl-S-methyl cysteine decreased [2-14C] acetate incorporation into fatty acid synthesis by 81%, 59%, 35% and 40%, respectively, at 2.0- 4.0 mmol/L concentrations. Alliin, γ-glutamyl-S-allyl cysteine, γ-glutamyl-S-propyl cysteine, S-allyl-N-acetyl cysteine, S-allylsulfonyl alanine, and S-methyl cysteine had no effect on fatty acid synthesis. The activities of lipogenic enzymes, fatty acid synthase (FAS) and glucose-6-phosphate dehydrogenase (G6PDH) were measured in cultured hepatocytes treated with the inhibitors. The activity of FAS in cells treated with 4.0 mmol/L SAC and SPC, respectively, was 32% and 27 % lower than that of non-treated cells. Neither SAC nor SPC affected G6PDH activity. The results indicate that SAC, SEC, and SPC inhibit lipid biosynthesis in cultured rat hepatocytes, and further suggest that these S-alk(en)yl cysteines of garlic impair triglyceride synthesis in part due to decreased de novo fatty acid synthesis resulting from inhibition on FAS. Whether tissue concentrations of active garlic components can achieve levels required to inhibit TG synthesis in vivo warrants further investigation.
4.2 INTRODUCTION

The cardiovascular protective effects of garlic have been extensively investigated in the past decades. It has been reported that garlic and various garlic preparations (e.g., aged garlic extract) reduce platelet aggregation in humans (1,2) and production of thromboxane B₂ in rats (3). Aged garlic extract has also been shown to decrease development of fatty streak and fibro fatty plaques in rabbits (4). Similarly, garlic powder supplementation in healthy adults attenuated age related increase in aortic stiffness by increasing aortic elasticity (5). A recent study with human volunteers has further demonstrated that aged garlic extract lowered the susceptibility of low-density lipoprotein (LDL) to oxidation (6). The potential reduction of risk for atherosclerosis and cardiovascular diseases by garlic has also been attributed primarily to its hypolipidemic property. However, the lipid lowering effects of garlic remain controversial. Although a number of studies reported that garlic lowered plasma total cholesterol, LDL-cholesterol, and triglyceride (TG) in animals (7-10) and in humans (11-14), several recent human intervention studies were unable to confirm the hypocholesterolemic effects of different garlic preparations (15-17). One of the possible explanations for the discrepancy may stem from the different ingredients of garlic or garlic preparations used in various studies (18). Moreover, the active components of garlic responsible for the putative lipid-lowering effects are ill defined (18). Raw garlic and garlic powder contain lipid-soluble thiosulfinates, thiosulfinate transformation products and sulfides (19). Among these compounds, allicin and vinyl dithiins have been suggested as potential lipid lowering agents (19). In addition, garlic in general and aged garlic extract in particular, contains significant amount of water-soluble organosulfur compounds including...
S-alk(en)yl cysteines and γ-glutamyl-S-alk(en)yl cysteines (19-21). We recently demonstrated that S-alk(en)yl cysteines and γ-glutamyl-S-alk(en)yl cysteines inhibited cholesterol biosynthesis in cultured rat hepatocytes (22). Among the compounds tested in cultured hepatocytes, three water-soluble S-alk(en)yl cysteines, i.e., S-allyl-cysteine (SAC), S-ethyl cysteine (SEC), and S-propyl cysteine (SPC), were the most potent inhibitors of cholesterol biosynthesis, achieving 42% to 55% maximal inhibition, albeit at high concentrations (22).

Despite extensive studies on understanding the effects of garlic on human lipid metabolism, little is known about the mechanisms underlying possible hypotriglyceridemic action of garlic or garlic preparations. A previous study from our laboratory demonstrated that garlic extracts inhibited [3H] glycerol incorporation into TG in rat cultured hepatocyte in the presence of acetate (10). When oleate was incubated with [3H] glycerol, such an inhibitory effect of the garlic extracts was no longer apparent (10). Consistent with these in vitro experiments, dietary supplementation of garlic decreased incorporation of [14C]acetate into fatty acids in various tissues of the rabbit (23). It is therefore reasonable to speculate that the inhibition of TG synthesis by garlic may in part be attributed to an impairment of fatty acid synthesis. The reduction of hepatic synthesis of TG could suppress very low-density lipoproteins (VLDL) synthesis in the liver and hence decrease plasma TG level (24). Therefore, the present study was undertaken to determine the inhibitory potency of garlic-derived organosulfur compounds on TG and fatty acid synthesis de novo in cultured rat hepatocytes. The results indicated that SAC and SPC at high concentrations (2.0–4.0 mmol/L) were potent inhibitors of fatty acid and TG synthesis. In addition, SAC and SPC
decreased the activity of fatty acid synthase (FAS) but had no effect on that of glucose-6-phosphate dehydrogenase (G6PDH).

4.3 MATERIALS AND METHODS

4.3.1 Animals

Male Sprague-Dawley rats (200-300 g) were obtained from Harlan Sprague-Dawley Co. (Indianapolis, IN) and fed a nonpurified diet (Purina Rat Chow, Ralston Purina, St. Louis, MO). The animals were housed individually in stainless steel cages at approximately 24°C and 50% relative humidity on a 12-h light/dark cycle (0600-1800). The animal protocol was approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

4.3.2 Hepatocyte isolation and cell culture

Hepatocytes were isolated from rats according to the method detailed previously (22). From each liver, 100-250 x 10^6 cells were obtained with a viability of 92-94% judging by trypan blue exclusion. The cells were resuspended in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units penicillin/mL, and 100 µg streptomycin/mL) to obtain 0.5-0.8 x 10^6 cells/mL of suspension. Aliquots (2 mL) of the suspension were plated in each well of a six-well culture plate (Becton Dickinson Labware, Franklin Lakes, NJ) and incubated at 37°C under an atmosphere of 95% air and 5% CO₂. After 4 h of incubation, nonadhering cells were removed and
discarded. Hepatocytes that adhered to the culture plate were refed with DMEM and incubated overnight for 16 h.

4.3.3 Metabolic study

At the end of the overnight incubation, cells were washed three times with 2 mL of FBS-free DMEM, followed by incubation with 2 mL of the same medium containing sodium salt of [2-\(^{14}\)C]acetate (specific activity, 37 MBq/mmol) and 0.5 mmol/L nonlabeled sodium acetate in the presence or absence of organosulfur compounds. Four hour after incubation, the medium was discarded and cells were harvested with 1.3 mL of ice-cold water by scraping with a cell scraper.

4.3.4 Lipid analysis

Cell suspension was mixed with 20 mL of chloroform/methanol (2:1, vol/vol) to extract lipids according to the method of Folch et al. (25) with minor modifications. For measurement of [2-\(^{14}\)C]acetate incorporation into fatty acid, the lipid extract was saponified with 6 mL of 3.75% methanolic KOH in a sealed ampule at 90 °C for 4 h (26). After the removal of nonsaponifiable fraction, the extracts were acidified with concentrated HCl, and the fatty acids were extracted with petroleum ether (b.p. 35-60 °C). For quantification of [2-\(^{14}\)C]acetate incorporation into TG and phospholipid, the lipid extracts were separated by thin-layer chromatography (TLC) on Silica Gel H coated plates (Analtech, Inc, Newark, DE) using hexane/diethyl ether/acetic acid (80:20:1, by vol) as developing solvent (27). The Silica gel bands corresponding to TG, and phospholipid were scraped into scintillation counting vials. The radioactivity of \(^{14}\)C-labeled products was measured by liquid scintillation
spectrometry (Beckman Model LS 3801; Beckman Instruments, Fullerton, CA). The specific activity of [2-\textsuperscript{14}C] acetate incorporation into fatty acid, TG and phospholipid was expressed as pmol acetate incorporated/mg cellular protein/4 h. Cellular protein was determined by the procedure of Lowry et al.\textsuperscript{(28)}. The relative rate of [2-\textsuperscript{14}C] acetate incorporation into fatty acid, TG, and phospholipid by cells treated with organosulfur compounds was expressed as percentage of control by calculating specific activity of treatment group/specific activity of control non-treatment group x 100. IC\textsubscript{50} (concentration required for 50\% of maximal inhibition) was calculated by regression and correlation analysis between substrate concentration and percentage inhibition.

4.3.5 Determination of enzyme activities

For measurement of enzyme activities, the cells were cultured in 60 mm-diameter culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ) plated with 3 x 10\textsuperscript{6} cells/dish. Cells were treated and incubated under the same conditions described above for metabolic study. After 4 h treatment, the medium was removed, and cells were washed three times with ice-cold phosphate-buffered saline. The dishes were placed on ice and immediately added 0.5 mL of potassium phosphate buffer (PPB,100 mmol/L, pH 7.4) containing sucrose, 250 mmol/L; EDTA,1 mmol/L; and dithiothreitol (DTT), 1 mmol/L (29, 30). The cells harvested by scraping were subjected to freezing and thawing three times and centrifuged at 20,000 x g for 30 min at 4°C (29). The supernatant obtained was used to determine the enzyme activities and protein concentration. FAS activity was determined spectrophotometrically by the method of Nepokroeff et al.\textsuperscript{(31)} in PPB (500 mmol/L, pH 7.0) containing malonyl CoA, 0.1 mmol/L; acetyl CoA, 0.05 mmol/L; NADPH, 0.1 mmol/L;
EDTA, 1 mmol/L; DTT, 5 mmol/L. The reaction was initiated by the addition of 0.1 mL malonyl CoA to a final volume of 1 mL, and the rate of oxidation of NADPH was monitored at 340 nm and 30°C with a Beckman DU®-50 LS 5801 spectrophotometer (Beckman Instruments, Fullerton, CA). G6PDH was measured by the method of Deutsch (32). The reaction mixture contained Tris buffer (100 mmol/L), pH 7.8, KCl (100 mmol/L), NADP (0.4 mmol/L), MgCl₂ (5 mmol/L), and glucose-6-phosphate (G-6-P) (5 mmol/L). The reaction was initiated by the addition of 0.1 mL of G-6-P to a final volume of 1 mL. The reduction of NADP was monitored spectrophotometrically at 340 nm. The specific activity of enzyme was defined as nmoles of NADPH oxidized/mg protein/min for FAS, and nmoles of NADP reduced/mg protein/min for G6PDH.

4.3.6 Materials

Culture media, FBS, penicillin and streptomycin were purchased from GIBCO (Gaithersburg, MD). Collagenase D was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). All water-soluble organosulfur compounds of garlic: SAC, SEC, S-methyl cysteine (SMC), SPC, γ-glutamyl-S-allyl cysteine (GSAC), γ-glutamyl-S-methyl cysteine (GSMC), γ-glutamyl-S-propyl cysteine (GSPC), S-allyl cysteine sulfoxide (i.e., Alliin), S-allyl-N-acetyl cysteine (SANC), S-allyl-mercaptocysteine (SAMC), and S-allylsulfonyl analine (SASA) were provided by Wakunaga of America Co., Ltd. (Mission Viejo, CA). Sodium [2⁻¹⁴C] acetate was obtained from Amersham Corp. (Arlington Heights, IL). All other chemicals of reagent grade were purchased from Sigma Chemical Co. (St. Louis, MO).
4.3.7 Statistics

Data are presented as means ± SEM (standard error of the mean). The comparisons of the test compounds were analyzed by analysis of variance (ANOVA) with the general linear model. When statistical significance was indicated by ANOVA, Dunnett test was applied to identify the significant difference between the treatment and control at $p <0.05$.

4.4 RESULTS

Eleven water-soluble organosulfur compounds present in garlic or aged garlic preparations were tested for inhibition potency on fatty acid synthesis in cultured hepatocytes. The rate of $[2^{-14}C]$ acetate incorporation into fatty acid in the untreated (control) group varied from 801 to 1127 pmol acetate/mg cellular protein/4 h among all experiments. The incorporation of $[2^{-14}C]$ acetate into fatty acids was taken as a measure of fatty acid synthesis. The rate of fatty acid synthesis in the untreated cells for individual experiment was arbitrarily defined as 100%. Among S-alk(en)y l cysteines, SAC, SEC, SPC but not SMC inhibited $[2^{-14}C]$ acetate incorporation into fatty acid with maximal inhibition of 35-80% (Figure 4.1). The inhibition was apparent at concentrations as low as 0.05 mmol/L for SAC and SEC, and 0.2 mmol/L for SPC (data not shown). The maximal inhibition was the highest by SPC followed by SAC and SEC. Among $\gamma$-glutamyl S-alk(en)y l cysteines, GSMC inhibited the rate $[2^{-14}C]$ acetate incorporation into fatty acid to a maximum of 40%, while GSAC and GSPC did not alter the rate of the incorporation (Figure 4.2). SAMC decreased the $[2^{-14}C]$ acetate incorporation into fatty acid by 35% at 0.05 mmol/L (Figure 4.2). Increasing concentration of SAMC to 2.0 and 4.0 mmol/L diminished fatty acid synthesis.
Other water-soluble compounds (i.e., alliin, SANC, and SASA) had no effect on the rate of [2-\(^{14}\)C] acetate incorporation into fatty acid within the range of concentrations (0.05 to 4.0 mmol/L) tested (data not shown).

The concentration-dependent inhibition shown in Figures 4.1 and 4.2 permitted us to calculate maximal inhibition and IC\(_{50}\) of water-soluble compounds on fatty acid synthesis. The maximal inhibition was the highest by SPC (81%) followed in decreasing order by SAC (59%), GSMC (40%), and SEC (35%), whereas the IC\(_{50}\) (expressed as mmol/L) was the lowest with GSMC (0.72) followed in increasing order by SEC (0.8), SPC (0.84) and SAC (0.91).

The effects of two potent inhibitors of fatty acid synthesis on the incorporation of \([^{14}\)C]acetate into TG and phospholipid were determined. SPC and SAC inhibited the rate of \([^{14}\)C]acetate incorporation into TG in a concentration dependent fashion exhibiting 51% and 43% maximal inhibition, respectively, at 4.0 mmol/L (Figure 4.3). A significant inhibition was observed at 0.05 mmol/L for SAC. In contrast, the inhibition by SPC was not apparent until the concentration was increased to 1.0 mmol/L. SAC and SPC inhibited \([^{14}\)C]acetate incorporation into phospholipid as well, but the inhibitory potency was less than that on TG (Figure 4.4). A significant inhibition of phospholipid production was not apparent until relatively high concentrations, i.e., 1.0 mmol/L for SAC and 2.0 mmol/L for SPC, were present in the incubation medium. The maximal inhibition was 43% for SAC, which was similar to that on TG (43%), and 30% for SPC, which was lower than that on TG (51%)
Figure 4.1 Inhibition of [2-14C]acetate incorporation into fatty acid by S-allyl cysteine (SAC), S-ethyl cysteine (SEC), S-methyl cysteine (SMC), and S-propyl cysteine (SPC) in primary rat hepatocyte culture. Data are expressed as a percentage of the control and represent means ± SEM of eight samples. *Statistically significant difference from controls at $p < 0.05$. 
Figure 4.2 Inhibition of $[2^{-14}\text{C}]$acetate incorporation into fatty acid by $\gamma$-glutamyl-S-allyl cysteine (GSAC), $\gamma$-glutamyl-S-methyl cysteine (GSMC), $\gamma$-glutamyl-S-propyl cysteine (GSPC), and S-allyl mercaptocysteine (SAMC) in primary rat hepatocyte culture. Data are expressed as a percentage of the control and represent means $\pm$ SEM of eight samples. *Statistically significant difference from controls at $p < 0.05$. 
Figure 4.3 Inhibition of [2-¹⁴C]acetate incorporation into triglyceride by S-allyl cysteine (SAC) and S-propyl cysteine (SPC) in primary rat hepatocyte culture. Data are expressed as a percentage of the control and represent means ± SEM of eight samples. *Statistically significant difference from controls at \( p < 0.05 \).
Figure 4.4 Inhibition of [2-^14^C]acetate incorporation into phospholipid by S-allyl cysteine (SAC) and S-propyl cysteine (SPC) in primary rat hepatocyte culture. Data are expressed as a percentage of the control and represent means ± SEM of eight samples. *Statistically significant difference from controls at $p < 0.05$. 
The marked inhibition on fatty acid biosynthesis led us to determine the effects of organosulfur compounds on the activities of lipogenic enzymes in cultured hepatocytes. The cells were incubated with SAC or SPC at the highest concentration (i.e., 4.0 mmol/L) that exhibited the maximal inhibition of fatty acid synthesis and at IC50. The activity of FAS in cells treated with 4.0 mmol/L SAC was 32% lower than that of non-treated cells (Table 4.1). SAC at IC50 (i.e., 0.91 mmol/l) depressed the FAS activity by 19%. On the other hand, SPC depressed the activity of FAS only at 4.0 mmol/L but not at IC50 (i.e., 0.84 mmol/L). Neither SAC nor SPC altered the activity of G6PDH at the concentrations tested (Table 4.1).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mmol/L)</th>
<th>FAS (^b)</th>
<th>G6PDH (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAC</td>
<td>0</td>
<td>3.71 ± 0.37</td>
<td>38.76 ± 3.43</td>
</tr>
<tr>
<td></td>
<td>0.91 (IC50)</td>
<td>3.00 ± 0.19*</td>
<td>37.12 ± 1.55</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>2.53 ± 0.32*</td>
<td>33.67 ± 1.81</td>
</tr>
<tr>
<td>SPC</td>
<td>0</td>
<td>4.06 ± 0.35</td>
<td>40.72 ± 1.96</td>
</tr>
<tr>
<td></td>
<td>0.84 (IC50)</td>
<td>3.57 ± 0.18</td>
<td>40.29 ± 1.00</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>2.94 ± 0.20*</td>
<td>37.36 ± 2.65</td>
</tr>
</tbody>
</table>

\(^a\)SAC, S-allyl cysteine, SPC, S-propyl cysteine.
\(^b\)The specific activity of enzyme is defined as nmoles of NADPH oxidized/mg protein/min for FAS and nmoles of NADP reduced/mg protein/min for G6PDH. Data represent means ± SEM of six samples.
*Statistically significant difference from nontreated group, \(p < 0.05\).
4.5 DISCUSSION

Hypertriglyceridemia is a risk factor independent of plasma level of cholesterol for cardiovascular disease (33). Further, this risk factor is not necessarily associated with decreased plasma level of high density lipoprotein cholesterol, and hence a reduction of TG-rich lipoprotein (i.e., VLDL) may attenuate the progression of coronary artery disease (34).

Evidence has suggested that garlic reduces the risk of cardiovascular disease by reducing plasma level of cholesterol (4,12,13, 23). Garlic may also decrease cardiovascular disease risk if it lowers plasma TG level. In fact, some clinical studies have shown that garlic or garlic preparations did lower plasma TG level (14,35). However, the mechanism underlying such a lowering action of garlic is unclear. Our previous study indicated that the TG-lowering effects of garlic extracts might stem in part from inhibition on hepatic TG synthesis (10). Furthermore, the reduction of TG synthesis was accompanied by depressed fatty acid synthesis in rat hepatocytes treated with various garlic extracts. Garlic is known to contain a large number of sulfur compounds (19). Although some of these compounds, such as SAC, have been shown to depress fatty acid synthesis (10), garlic constituents foremost responsible for inhibiting TG synthesis are yet to be determined. The present study revealed that sulfur containing cysteine derivatives (i.e., SAC and SPC) were potent inhibitors of TG synthesis in vitro. Moreover, SAC and SPC were found to be the most potent inhibitors of fatty acid synthesis among S-alk(en)yl cysteines (e.g., SAC, SEC, SMC, and SPC) and γ-glutamyl-S-alk(en)yl cysteines (e.g., GSAC, GSMC, and GSPC) tested. These observations are consistent with our earlier study suggesting that garlic decrease hepatic TG production by inhibiting fatty acid synthesis (10). In addition, the current study further identified SAC and
SPC as the most active garlic components for inhibition of fatty acid synthesis. The present data, however, do not exclude the possibility that garlic constituents affect distal pathway of TG synthesis. For example, whether SAC, SPC, or other cysteine derivatives alter activities of glycerolipid synthesizing enzymes such as glycerol-3-phosphate acyltransferase, monoacylglycerol acyltransferase, phosphatidate phosphohydrolase, and diacylglycerol acyltransferase is not known (36). It is worthwhile to note that the incorporation of [2-14C] acetate into phospholipid was also inhibited in a similar manner as TG synthesis. Since phospholipid synthesis shares most acyltransferases and phosphatidate phosphohydrolase with TG synthesis, this finding tends to support the notion that the inhibition of TG synthesis by sulfur compounds may not involve esterification steps catalyzed by the acyltransferases.

Another important finding was that organosulfur compounds decreased fatty acid synthesis (Figures 4.1 and 4.2), and the patterns of inhibition were similar to those on cholesterol synthesis as reported previously (22). Three S-alk(en)yl cysteines, SAC, SEC and SPC, but not SMC, significantly decreased fatty acid synthesis with maximal inhibition of 59%, 35% and 81%, respectively. Among γ-glutamyl S-alk(en)yl cysteines, GSMC reduced [2-14C] acetate incorporation into fatty acid, while GSAC and GSPC did not affect fatty acid synthesis. Consistent with unaltered rate of cholesterol synthesis, Alliin, SANC and SASA did not change [2-14C] acetate incorporation into fatty acid, whereas SAMC containing two sulfur atoms markedly decreased fatty acid synthesis. However, unlike other water-soluble compounds, SAMC is highly cytotoxic as reported earlier in this laboratory (22). Therefore, the reduction on [2-14C] acetate incorporation into fatty acid by SAMC may result from cytotoxicity. The decrease of [2-14C] acetate incorporation into fatty acid by other compounds, i.e., SAC, SEC, SPC, and GSMC, may result from impairment in the enzyme or
enzymes regulating fatty acid synthetic pathways. In fact, the present study with cultured hepatocytes demonstrated that SAC and SPC incubated at the concentration (4.0 mmol/L) that caused maximal inhibition of fatty acid synthesis decreased the activity of FAS by 32% and 27%, respectively, when compared with non-treated cells. Neither SAC nor SPC exhibited inhibitory effect on the activity of G6PDH. These data suggest that the sulfur-containing compounds depress fatty acid synthesis by decreasing the activity of FAS, the rate limiting enzyme for fatty acid synthesis. Earlier animal studies by other investigators showed that garlic-supplemented diets decreased activities of not only lipogenic FAS but also G6PDH (7-9). The reason for the discrepancy in G6PDH activity observed between the present study and that of others (7-9) is not readily understood. However, it is important to point out that SAC and SPC isolated from garlic were used in the present in vitro experiment as compared with various garlic extracts fed to animals (7-9). Garlic extracts used in the studies contained not only SAC and SPC but also other water-soluble and fat-soluble sulfur compounds (37). Thus, animal studies are warranted to further delineate the differential effects of individually isolated compounds as compared with garlic extracts on lipogenic enzymes. Also, it should be stressed that whether SAC, SEC, SPC, and other sulfur containing compounds interfere with fatty acid chain elongation by [2-14C] acetate and hence play any role in the observed inhibition of TG synthesis remains to be ascertained.

Finally, the present study using rat cultured hepatocytes demonstrated that water-soluble organosulfur compounds of garlic, especially SAC and SPC, inhibited TG synthesis by depressing fatty acid synthesis de novo. The inhibition of fatty acid synthesis, on the other hand, was associated with decreased activity of FAS. Overproduction of TG rich-VLDL in liver is known to induce hypertriglyceridemia (38). It is therefore tempting to speculate that
decreased hepatic TG synthesis by water-soluble organosulfur compounds may explain in part the hypotriglyceridemic effect of garlic reported previously (14,35). However, one must be cautious about the extrapolation of in vitro data to in vivo situation. It is important to stress that although maximal inhibition of fatty acid and TG synthesis was obtained at 4.0 mmol/L of alk(en)yl cysteines (i.e., SAC and SPC), significant inhibition of fatty acid synthesis was detected at a concentration as low as 0.05 mmol/L in the present in vitro study (Figures 4.1 and 4.3). The inhibition of TG synthesis by SAC was also apparent in the same concentration range of 0.05 to 4.0 mmol/L (Figure 4.3). It is essential to know whether these concentrations can be achieved under in vivo conditions. Unfortunately, there is no such information available for humans. Nonetheless the present study in vitro clearly indicates that fatty acid and TG synthesis are inhibited by SAC and other alk(en)yl cysteines in a concentration-dependent manner. These findings further suggest that any effect of inhibition of the sulfur compounds on cholesterol synthesis and plasma cholesterol is likely dependent upon their tissue concentrations. Although garlic and garlic preparations have been shown to reduce (11-14, 39) or have no effect (15-17) on plasma concentration of cholesterol and TG, the reported reduction of plasma concentration of cholesterol was mild with a mean of 9% according to the meta analysis of Warshafsky et al. (39). Whether the low magnitude of the reduction in plasma cholesterol concentration is attributed to low tissue level of potential active components of garlic remains to be established. The reason for the contradictory observations of the effect of garlic on cholesterol is uncertain, it may be explained in part by nonstandardized experimental designs. In addition the present study further suggests the following parameters as important determinants of whether garlic affects plasma lipids: (i) the type of garlic preparations, e.g., garlic powder, garlic oil, or aged garlic extract, used in
different studies; (ii) active components, e.g., lipid-soluble allicin and vinyl dithiin oils, or water-soluble alk(en)yl cysteines, available in garlic preparations; and (iii) if the amount of active components is sufficient to achieve the plasma concentration required for inhibition of hepatic cholesterol and TG biosynthesis.
4.6 REFERENCES


CHAPTER 5

REGULATION OF HMG-COA REDUCTASE BY ORGANOSULFUR COMPOUNDS OF GARLIC IN PRIMARY CULTURES OF RAT HEPATOCYTES
5.1 ABSTRACT

The study was undertaken to elucidate the mechanism by which water-soluble organosulfur compounds of garlic inhibit hepatic cholesterol biosynthesis. In cultured rat hepatocytes, treatment of cells with S-alk(en)yl cysteines, i.e., S-allyl cysteine (SAC), S-ethyl cysteine (SEC), and S-propyl cysteine (SPC) inhibited cholesterol synthesis from $[^{14}\text{C}]$acetate (Liu and Yeh (2000) Lipids 35, 197-203) but not from $[^{14}\text{C}]$mevalonate, suggesting that the point of regulation is at 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase. Consistent with this notion, the activity of HMG-CoA reductase in the cells treated with SAC, SEC, and SPC was 30% to 40% lower than that of non-treatment group. Abundance of mRNA coded for HMG-CoA reductase was not altered by the sulfur compounds. Neither did these compounds change protein concentration of HMG-CoA reductase. The expressed (E) and total (T) activities of HMG-CoA reductase were then determined and the ratios of E/T were used to estimate the phosphorylation state of the enzyme. SAC, SEC, and SPC reduced the ratios of E/T by 18% to 29% resulting primarily from decreased expressed activity. The activity of HMG-CoA reductase was also measured at a lower dithiothreitol concentration and pre-incubation of microsomes with phosphatase to test the involvement of thiol redox status. Under the experimental conditions, SAC was the only compound found to suppress the activity of the enzyme. The results suggest that the organosulfur compounds decrease the activity of HMG-CoA reductase by phosphorylation of the enzyme, but not by alteration of gene expression. In addition, SAC appears to further inhibit the activity of HMG-CoA reductase by increasing sulfhydryl oxidation of the enzyme.
5.2 INTRODUCTION

Garlic and organosulfur compounds of garlic have been shown to decrease hepatic cholesterol synthesis (1-6) which may explain in part the hypocholesterolemic effect of garlic in humans and animals (7-10). Three water-soluble S-alk(en)yl cysteines, i.e., S-allyl-cysteine (SAC), S-ethyl cysteine (SEC), and S-propyl cysteine (SPC) were the most potent inhibitors of cholesterol synthesis, achieving 42% to 55% maximal inhibition in cultured hepatocytes (6). However, the underlying mechanisms have not been fully elucidated. In vitro study demonstrated that water-soluble garlic extracts reduced hepatocyte cholesterol synthesis when $[^{14}\text{C}]$acetate but not $[^{14}\text{C}]$mevalonate was used as a precursor, indicating a potential regulation at 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (1). In fact, animal studies have shown that garlic-supplemented diets decreased hepatic activity of HMG-CoA reductase (7, 11). However, the regulatory mechanisms of HMG-CoA reductase activity by garlic compounds are still unclear.

The regulation of HMG-CoA reductase may exert at the transcriptional or post-transcriptional level (12-14). Post-transcriptional regulation could be at the levels of translation, protein degradation, and catalytic efficiency including phosphorylation and thiol redox status of the enzyme (15, 16). In cultured Chinese hamster ovary (CHO) cells, sterols were shown to regulate the enzyme mainly at the level of transcription whereas non-sterols exerted regulation at the post-transcriptional level including translation and protein degradation (13). Studies with CHO and baby hamster kidney cells indicate that the N-terminal 8-transmembrane domain of the reductase is required for the degradation of the enzyme (17, 18). HMG-CoA reductase is inactivated by phosphorylation (13). This reaction
is catalyzed by a protein kinase family, AMP-activated protein kinase (19), and the inactivated enzyme can be reactivated by the action of a phosphatase (20). In addition, HMG-CoA reductase can be inactivated by sulfhydryl oxidation and reactivated by high concentrations of thiols such as dithioerythritol and dithiothreitol (21, 22).

This study was undertaken to determine the effects of S-alk(en)yl cysteines on cholesterol synthesis from [14C]mevalonate and the activity of HMG-CoA reductase, and to investigate the possible regulatory mechanisms of HMG-CoA reductase activity by these compounds in cultured rat hepatocytes. The mRNA and protein levels of HMG-CoA reductase were determined after treatment with various compounds. The possible involvement of phosphorylation and thiol redox status of the enzyme by the garlic compounds was explored as well. The results suggest that sulfur compounds of garlic inhibit cholesterol synthesis primarily by depressing HMG-CoA reductase activity resulting from phosphorylation or sulfhydryl oxidation of the enzyme.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Male Sprague-Dawley rats (200-300 g) were obtained from Harlan Sprague-Dawley Co. (Indianapolis, IN) and fed a nonpurified diet (Purina Rat Chow, Ralston Purina, St. Louis, MO). The animals were housed individually in stainless steel cages at approximately 24°C and 50% relative humidity on a 12-h light/dark cycle (1000-2200). The animal protocol was approved by The Pennsylvania State University Institutional Animal Care and Use Committee.
5.3.2 Hepatocyte isolation and culture

Hepatocytes were isolated from rats according to the method of Berry and Friend (23) as modified by Seglen (24), and detailed in Chapter 3. From each liver, 100-250 x 10^6 cells were obtained with a viability of 92-94%, judging by trypan blue exclusion. The cells were resuspended in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units penicillin/mL plus 100 µg streptomycin/mL) to obtain approximately 0.75 x 10^6 cells/mL of suspension. Two-milliliter aliquots of cell suspension were plated in each well in a six-well culture plate (Becton Dickinson Labware, Franklin Lakes, NJ) for the measurement of [14C]mevalonate incorporation into cholesterol. Four-milliliter aliquots of the suspension were plated in each 60 mm-diameter culture dish (Becton Dickinson Labware, Franklin Lakes, NJ) in order to get a sufficient number of cells for determining the activity of HMG-CoA reductase and the levels of mRNA and protein of the enzyme. The plates or dishes were then incubated at 37°C under an atmosphere of 95% air and 5% CO₂. After 4 h of incubation, nonadhering cells were removed and discarded. Hepatocytes that adhered to the culture plates and dishes were refed with DMEM and incubated overnight for 16 h.

5.3.3 Determination of [14C]mevalonate incorporation into cholesterol

At the end of the overnight incubation, cells cultured in the plate were washed three times with 2 mL of FBS-free DMEM. The cells were then incubated with 2 mL FBS-free medium containing [2-14C] mevalonate (0.24 µCi/mL) in the absence or presence of S-allyl cysteine, S-ethyl cysteine, or S-propyl cysteine at IC₅₀ and 4 mmol/L. Four hours after the incubation, the cells were harvested for extraction of cholesterol (6). The radioactivity of
[\textsuperscript{14}C]-labeled cholesterol was determined by liquid scintillation spectrometry (Beckman Model LS 3801, Beckman Instruments, Fullerton, CA). The specific activity of cholesterol synthesis was expressed as pmol mevalonate incorporation into cholesterol/mg cellular protein/4h. The relative rate of cholesterol synthesis by cells treated with organosulfur compounds was expressed as percentage of control (control was set as 100%) (6).

5.3.4 Preparation of hepatocyte microsomes

Cells cultured in the dish were washed three times with 2 mL of FBS-free DMEM after the overnight incubation and treated with 4 mL FBS-free DMEM in the absence or presence of test compounds at IC\textsubscript{50} and/or 4 mmol/L. After 4 h incubation, cells were washed twice with 2 mL ice-cold buffer A (50 mmol/L Tris-HCl and 150 mmol/L NaCl, pH 7.4). The dishes were placed on ice and immediately added 1 mL of buffer A. The cells were then harvested by scraping. The content of each dish was rinsed with 1 mL buffer A and added to the suspension of scraped cells. The cell suspension was centrifuged at low speed (900 \times g, 5 min at 4\textdegree C) and the resulting supernatant was discarded (25). The cell pellet was dissolved in 1 ml buffer B (50 mmol/L Tris-HCl, 0.3 mol/L sucrose, 50 mmol/L NaCl, 10 mmol/L EDTA, and 10 mmol/L dithiothreitol (DTT), pH 7.4) and sonicated using a sonifier (Branson Model 250, Branson Ultrasonics Corporation, Danbury, CT). The sample was then centrifuged at 12,000 \times g for 15 min at 4\textdegree C. The pellet was discarded and the supernatant was recentrifuged at 110,000 \times g for 1.5 h at 4\textdegree C. The resulting microsomal pellet was suspended in 100 \mu L buffer C (20 mmol/L imidazol-HCl, 10 mmol/L DTT) and aliquots were immediately used for determination of HMG-CoA reductase activity (26-28).
5.3.5 HMG-CoA reductase activity

HMG-CoA reductase activity was measured by radiochemical assay using thin-layer chromatography (TLC) as described by Goldstein et al. (25). Aliquots of microsome (20-100 µg of protein in 50 µL buffer C) were mixed with 100 µL of buffer D (200 mmol/L potassium phosphate, 12 mmol/L DTT, and 4 mmol/L NADPH, pH 7.4) and 40 µL of water. Ten µL of HMG-CoA (624 µmol/L, specific radioactivity 20,000dpm/nmol) used as the substrate was added to initiate the reaction. The reaction mixture was incubated for 60 min at 37°C and terminated by addition of 20 µL of 5N HCl. After addition of [5-3H]mevalonolactone as an internal standard, the mixture was incubated for another 30 min at 37°C to lactonize [14C]mevalonate product to [14C]mevalonolactone. Mevalonolactone was isolated by TLC (25), and the radioactivity of [14C]mevalonolactone and [3H]mevalonolactone was counted by a liquid scintillation system (Beckman Model LS 3801, Beckman Instruments, Fullerton, CA). The percentage of added [3H]mevalonolactone recovered during the assay was calculated and used for correction of [14C]mevalonolactone formed. The activity of HMG-CoA reductase was expressed as picomoles of [14C]mevalonate formed per mg of microsomal protein per min (pmol/mg protein/min). The radioactivity counting from each sample ranged from 1,500 to 2,000 dpm that represented 1.2-1.6% of the radioactivity provided in the assay medium. For determination of the expressed activity of HMG-CoA reductase, the microsomes were prepared in a buffer similar to buffer B except NaCl was replaced by NaF, and assayed as above. For determination of the total activity of HMG-CoA reductase, microsomes was prepared in buffer B and the activity was measured as above except that microsomes were pre-incubated for 60 min in the presence of 10 U of phosphatase (26, 29).
5.3.6 Real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

For measurement of mRNA abundance, cells in the dish were treated with test compounds at 4.0 mmol/L in 4 mL FBS-free DMEM after the overnight incubation. Four hours after the treatment, cells were collected and total RNA was extracted with RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. HMG-CoA reductase mRNA was quantified by real-time RT-PCR with a Perkin-Elmer/Applied Biosystems Division (PE/ABD) Prism 7700 sequence detection system (Foster City, CA, 30). RT-PCR reaction was performed with the AmpliTaq Gold polymerase (PE/ABD, Foster City, CA) with 20 ng total RNA for each reaction. For quantifying a particular mRNA with real-time RT-PCR, a fluorogenic probe and two primers were designed and synthesized. The internal oligonucleotide probe was labeled with a fluorescent dye, carboxyfluorescein-aminohexyl amidite (FAM), at the 5’ end and black hole quencher (BHQ) dye at the 3’ end (Biosearch Technologies, Novato, CA). When both dyes were present in the intact probe, BHQ acted as a quencher for FAM by absorbing at the FAM emission spectra. When the internally hybridized probe was degraded by the 5’ exonuclease activity of Taq polymerase during the course of PCR, these two dyes were separated in solution, resulting in subsequent increase in the level of fluorescence in the reaction mixture. Thus, the amount of fluorescence released during each amplification cycle was proportional to the amount of specific PCR products generated in that cycle. The 18S RNA was amplified at the same time and used as an internal control. The threshold cycle (Ct) values for 18S RNA and samples were calculated by PE/ABD computer software. Ct was determined by identifying the cycle number at which the fluorescent dye emission intensities rose above background noise and was obtained at the most exponential phase of the reaction. Relative transcript levels were
calculated as $x = 2^{-\Delta\Delta C_t}$, in which $\Delta\Delta C_t = \Delta E - \Delta C$, and $\Delta E = C_{\text{experiment}} - C_{\text{control}}$. $\Delta C_{18s}$.

The primers and probe of HMG-CoA reductase were designed according to GenBank Accession Number X55286 using PE/ABD Primer Express software, which is specifically designed for the selection of primers and probes. The forward and reverse primers for rat HMG-CoA reductase mRNA were 5'-ACCGTGGGTGGGAC-3' (17 nucleotides) and 5'-GCCCTTTGAACACCTAGCATC-3' (21 nucleotides), respectively. The fluorogenic internal probe was 5'-(FAM)ACCTTCTACCTCAGCAAGCCTGCCTGC(BHQ)-3' (27 nucleotides).

5.3.7 Cell lysate preparation for immunoblot analysis

Hepatocytes cultured in the dish were treated as described in RT-PCR. Four hours after the treatment with the test compounds, the medium was removed and cells were washed twice with 2 mL ice-cold PBS buffer. The dishes were placed on ice and 1 mL of buffer A was added immediately. Cells were harvested by scraping. The content of each dish was rinsed with 0.5 mL buffer A and added to the suspension of scraped cells. The cell pellet was prepared as described earlier in the microsome preparation (25). After the centrifugation, the cell pellet was then dissolved in 80 µL lysis buffer and incubated on ice for 60 min. The lysis buffer consisted of 1% Nonidet P-40, 0.1% SDS, 1mmol/L NaOV3, and protease inhibitor mixture prepared according to the manufacturerís instructions (Roche Molecular Biochemical, Indianapolis, IN) in PBS. The cell lysate was centrifuged at 10,000 × g for 20 min at 4°C, and the supernatant was taken as the total cell lysate. The protein concentration of cell lysate was determined by the procedure of Lowry et al. (31).
5.3.8 Immunoblot analysis

Polyclonal antisera to a proteolytic fragment containing the catalytic domain of rat HMG-CoA reductase were generated in rabbits (32) and were a generous gift from Dr. Ness (University of South Florida). The reductase antisera recognize a 100-kDa band as the dominant species (33). However, if the reductase was degraded by proteolysis, other bands might appear at about 70-kDa or less (32). For immunoblot analysis, 20 µL sample buffer (30 mmol/L Tris-HCl, 1% SDS, 0.1 mol/L sucrose, 8 mol/L urea, 5% β-mercaptoethanol, 0.005% bromophenol blue, pH 6.8) was added to 50 µg protein of cell lysate. The sample was then boiled for 5 min and subjected to gel electrophoresis on a 7.5% SDS-polyacrylamide gel (33). The separated protein was electrophoretically transferred to PVDF-plus membrane purchased from Micron Separations, Inc.(Westboro, MA). The membranes were blocked with 5% Carnation nonfat dry milk. They were then incubated at room temperature for 2 h with a 1:5000 dilution of HMG-CoA reductase antisera. Immunoreactive protein was detected using the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ). The relative level of HMG-CoA reductase immunoreactive protein was determined using a phosphoimager (15, 33).

5.3.9 Materials

Culture media, FBS, penicillin and streptomycin were purchased from Life Technologies (Rockville, MD). Collagenase D was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Alkaline phosphatase was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Three water-soluble S-alk(en)yl cysteines of garlic, i.e., SAC, SEC, and SPC, were provided by Wakunaga of America Co., Ltd. (Mission
Viejo, CA). [2-14C]mevalonate, dibenzylethylendiamine salt and [3-14C]HMG-CoA were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). [5-3H]mevalonolactone was purchased from American Radiolabeled Chemicals, Inc.(St. Louis, MO). All other chemicals of reagent grade were purchased from Sigma Chemical Co.(St. Louis, MO).

5.3.10 Statistics

Data are presented as means ± standard error of the mean (SEM). The comparisons of the test compounds were analyzed by analysis of variance (ANOVA) with general linear model. When statistical significance was indicated by ANOVA, the Dunnett test was applied to identify the significant difference between the treatment and control at $p < 0.05$.

5.4 RESULTS

In an earlier study, we showed a significant reduction on the [14C]acetate incorporation into cholesterol by S-alk(en)yl cysteines including SAC, SEC, and SPC (6). Therefore, in the present study, the incorporation of [14C]mevalonate into cholesterol was measured in cultured hepatocytes to determine the regulation of cholesterol synthesis by S-alk(en)yl cysteines. The average rate of [14C]mevalonate incorporation into cholesterol was 1878 pmol/mg cellular protein/4h for control and ranged from 1800 to 1944 pmol/mg cellular protein/4h among the treatment groups. The rate of [14C]mevalonate incorporation into cholesterol for control was set to 100%, and the values for the treatment groups were expressed as percentage of control. None of S-alk(en)yl cysteines, i.e., SAC, SEC, and SPC, altered the incorporation of [14C]mevalonate into cholesterol when the relative rate of the
treatment groups was compared with that of control (Table 5.1) suggesting the regulatory point by the compounds might be at HMG-CoA reductase. The activity of HMG-CoA reductase was then determined in the cultured cells treated with S-alk(en)yl cysteines at the concentrations of IC<sub>50</sub> and 4 mmol/L. The concentrations of IC<sub>50</sub> for SAC, SEC, and SPC were 0.61, 0.58, and 0.72 mmol/L, respectively, as determined previously (6). The activity of HMG-CoA reductase was decreased 35%, 27%, and 33% by SAC, SEC, and SPC at concentrations of IC<sub>50</sub>, respectively (Table 5.1). The activity of HMG-CoA reductase was reduced 30% to 41% at the concentration of 4.0 mmol/L by the S-alk(en)yl cysteines (Table 5.1). The percent inhibition of SAC on the activity of HMG-CoA reductase at 4.0 mmol/L was higher than that of SEC.

Table 5.1. Effects of SAC, SEC, and SPC on [14C]mevalonate incorporation into cholesterol and activity of HMG-CoA reductase in cultured hepatocytes<sup>a</sup>

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration&lt;sup&gt;b&lt;/sup&gt; (mmol/L)</th>
<th>[14C]mevalonic acid incorporation into cholesterol&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Activity of HMG-CoA reductase&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAC</td>
<td>0</td>
<td>100.0 ± 2.9</td>
<td>36.9 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>0.61 (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>98.3 ± 3.4</td>
<td>24.0 ± 1.0*</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>97.2 ± 4.1</td>
<td>21.7 ± 1.3*</td>
</tr>
<tr>
<td>SEC</td>
<td>0</td>
<td>100.0 ± 2.9</td>
<td>40.0 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>0.58 (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>101.5 ± 3.2</td>
<td>29.4 ± 0.9*</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>103.4 ± 4.4</td>
<td>28.1 ± 2.0*</td>
</tr>
<tr>
<td>SPC</td>
<td>0</td>
<td>100.0 ± 2.9</td>
<td>40.0 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>0.72 (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>100.8 ± 2.4</td>
<td>26.8 ± 0.7*</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>95.8 ± 2.2</td>
<td>25.3 ± 1.7*</td>
</tr>
</tbody>
</table>

<sup>a</sup>SAC, S-allyl cysteine; SEC, S-ethyl cysteine; SPC, S-propyl cysteine.
<sup>b</sup>IC<sub>50</sub>, concentration required for 50% of maximal inhibition.
<sup>c</sup>Specific activity expressed as pmol/mg cellular protein/4 h for control was set at 100% and the values for treated groups were expressed as percentage of control. Specific activities for control groups ranged from 1700 to 2000 pmol/mg protein/4 h. Data represent means ± SEM of ten samples.
<sup>d</sup>Specific activity was expressed as pmol/mg protein/min. Data represent means ± SEM of six samples. *Statistically different from non-treated group at p < 0.05.
In order to further elucidate the possible regulatory mechanisms of HMG-CoA reductase activity by organosulfur compounds of garlic, the levels of HMG-CoA reductase mRNA and protein were determined in cultured hepatocytes. As shown in Figure 5.1, the relative mRNA level of HMG-CoA reductase was not different among the treatment groups and control. Figure 5.2 shows the result of immunoblot analysis of HMG-CoA reductase. Molecular weight markers (118, 85, and 62 kDa) were indicated on the left side of the picture. The upper band between 85 to 118 kDa represented HMG-CoA reductase, and the number above each band was the percentage of density relative to the total density of eight lanes. The lower band around 70 kDa was the degraded product of the enzyme (32). As shown, the amount of HMG-CoA reductase protein remained unchanged by the treatment of SAC, SEC, and SPC. (Figure 5.2). Clearly, S-alk(en)yl cysteines had no effect on the abundance of HMG-CoA reductase mRNA and the amount of the enzyme protein.

Activation/deactivation of HMG-CoA reductase by phosphorylation/dephosphorylation was examined. The expressed (E) and total (T) activities of HMG-CoA reductase were measured in the presence or absence of phosphatase, respectively. The phosphorylation state of the enzyme was estimated by the ratios of E/T (34). The ratios of E/T were 18% to 29% lower in cells treated with three S-alk(en)yl cysteines than that of non-treatment group (Figure 5.3). The ratios of E/T showed no significant difference between SAC and SPC treatment, but the ratio of SPC treated group was lower than that of SEC (Figure 5.3). The decreased E/T ratios primarily resulted from decreased expressed activity because total activity remained unchanged (ranged from 91 to 94 pmol/mg protein/min) among the treatment and control groups.
Figure 5.1 The levels of HMG-CoA reductase mRNA in cultured hepatocytes after organosulfur compound treatment. The mRNA levels were normalized to 18S. Relative mRNA abundance for control was arbitrarily set to 1 unit. Experimental groups were treated with S-allyl cysteine (SAC), S-ethyl cysteine (SEC), and S-propyl cysteine (SPC) at 4 mmol/L. Values represent means ± SEM for five samples.
Figure 5.2 Immunoblot analysis of HMG-CoA reductase in S-alk(en)yl cysteine-treated hepatocytes. Cells were treated with or without S-allyl cysteine (SAC), S-ethyl cysteine (SEC), and S-propyl cysteine (SPC) at 4 mmol/L. Molecular weight markers (118, 85, and 62 kDa) were indicated on the left side of the picture. Control: lane 1-2; SAC: lane 3-4; SEC: lane 5-6; SPC: lane 7-8. Number represents percentage of upper band density relative to total density of eight lanes.
Figure 5.3 The effects of S-allyl cysteine (SAC), S-ethyl cysteine (SEC), and S-propyl cysteine (SPC) on E/T ratios of HMG-CoA reductase in cultured hepatocytes. Cells were treated with or without SAC, SEC, SPC at 4.0mmol/L. Values represent means ± SEM for four samples. Values not sharing a common superscript differ at $p < 0.05$. 
The possible role of thiol redox status in regulating HMG-CoA reductase activity was also tested. Figure 5.4 illustrated the different HMG-CoA reductase activities measured in the presence of different DTT concentrations in buffer D (see Materials and Methods, section 5.3.6) and different phosphatase concentrations. As shown in this figure, the activities of HMG-CoA reductase increased with increasing DTT concentrations from 4 mmol/L to 12 mmol/L (Figure 5.4A). However, there was no further increase of the enzyme activity from 12 mmol/L to 16 mmol/L DTT (Figure 5.4A). The activity of HMG-CoA reductase was also enhanced by increasing phosphatase from 5 U to 10 U, and there were no differences in HMG-CoA reductase activities at 10 U, 15 U, and 20 U concentrations (Figure 5.4B). Thus, as described in the method, the activity of HMG-CoA reductase was routinely measured at 12 mmol/L DTT, whereas the total activity of the enzyme was determined with microsomes pre-incubated with 10 U phosphatase in the presence of 12 mmol/L DTT. In order to test whether the organosulfur compounds affect the thiol redox status, DTT at 8 mmol/L and phosphatase at 10 U were employed in determining the enzyme activity. Under these conditions, phosphorylation of the enzyme was diminished in the presence of phosphatase. Hence, any effect on the enzyme was assumed to be a result of the change in thiol redox status of the enzyme. As stated above, the total activity of HMG-CoA reductase measured in the presence of 10 U phosphatase and 12 mmol/L DTT was not altered by S-alk(en)yl cysteines. However, the activity measured after pre-incubation of microsomes with 10 U phosphatase in the presence of 8 mmol/L DTT was reduced 10% by SAC, but not by SEC or SPC (Figure 5.5).
Figure 5.4 The changes of HMG-CoA reductase activity with different concentrations of DTT in buffer D (A) and amounts of phosphatase (B). Values represent means ± SEM for four samples. Values not sharing a common superscript differ at $p < 0.05$. 
Figure 5.5 The activity of HMG-CoA reductase measured in lower concentration (8 mmol/L) of DTT and pre-incubated microsomes with phosphatase (10 U). Cells were treated with or without S-allyl cysteine (SAC), S-ethyl cysteine (SEC), and S-propyl cysteine (SPC) at 4.0 mmol/L. Values represent means ± SEM for four to five samples. *Statistically different from control at $p < 0.05$. 

HMG-CoA reductase activity (pmol/mg protein/min)
5.5 DISCUSSION

We previously reported that organosulfur compounds inhibited $[^{14}C]$acetate incorporation into cholesterol in cultured rat hepatocytes (6). The present study, however, showed that the rate of $[^{14}C]$mevalonate incorporation into cholesterol remained the same whether cells were treated with or without S-alk(en)yl cysteines. These results suggest that the sulfur compounds regulate cholesterol synthesis at the point of HMG-CoA reductase. This notion was supported by the decreased activity of HMG-CoA reductase in cells treated with S-alk(en)yl cysteines. The activity of HMG-CoA reductase was depressed 41% by SAC, 37% by SPC, and 30% by SEC. The reduction of the enzyme activity was consistent with the findings in other in vitro and animal feeding studies (1, 7, 11).

However, the mechanisms by which S-alk(en)yl cysteines decreased the activity of HMG-CoA reductase are not clear. The regulation of HMG-CoA reductase could be at the level of gene expression including transcription of the gene and translation of mRNA (13, 16). The enzyme could also be activated or deactivated by the interchange of thiol-disulfide, and dephosphorylation-phosphorylation (15, 35). The induction and repression of the mRNA for HMG-CoA reductase had been observed in Chinese hamster ovary cells that were cultured in the presence or absence of sterols (13). Studies have also shown that the levels of mRNA and/or protein of hepatic HMG-CoA reductase were altered by nutritional status and hormones such as insulin and glucagon (15, 36-38). Interestingly, the sulfur compounds tested in present study did not alter the abundance of mRNA coded for HMG-CoA reductase. Neither did these compounds affect protein concentration of HMG-CoA reductase. It was apparent that S-alk(en)yl cysteines had no effect on gene expression of HMG-CoA reductase in the cultured rat hepatocytes under the present experimental conditions.
HMG-CoA reductase activity is known to be modulated by phosphorylation and dephosphorylation (39, 40). It has been reported that hepatic HMG-CoA reductase was inactivated in vitro when microsomes were incubated with cytosol in the presence of ATP and Mg\(^{2+}\) (41). The inactivated reductase was reactivated with a purified enzyme from rat liver, and the reactivation was inhibited by sodium fluoride (NaF), an inhibitor of phosphatases, suggesting that the reactivation involved a dephosphorylation of a phosphorylated enzyme (40). Direct evidence for the involvement of phosphatase in the reactivation came from studies demonstrating that reactivation of the enzyme followed incubation of the inactivated reductase with partially purified hepatic phosphoprotein phosphatase (20, 39). The phosphorylation state of HMG-CoA reductase may be estimated by determining the ratio of expressed (E) to total (T) activity of the enzyme. A strong linear relationship between the ratio of E/T and the fraction of dephosphorylated enzyme established by Parker et al. (34) suggests that E/T ratio is a valid index of the HMG-CoA reductase phosphorylation state. In other words, the E/T ratio is an accurate representation of the percent of microsomal reductase in the dephosphorylated mode (34, 42); the higher the ratio, the more the enzyme is dephosphorylated. The expressed activity, measured in microsomes prepared with NaF and EDTA, represents only the enzyme present in the dephosphorylated form. The total activity, measured in microsomes prepared in the absence of NaF and pre-incubated with phosphatase to induce maximal activation of the enzyme, is an indirect evaluation of the total enzyme presented in dephosphorylated and phosphorylated forms (29). Therefore, we used the E/T (dephosphorylated/(dephosphorylated + phosphorylated)) ratio as an index of the HMG-CoA reductase phosphorylation state in this study. The E/T ratios were reduced 29%, 21%, and 18% by SPC, SAC, and SEC,
respectively. The results indicated that S-alk(en)yl cysteines increased phosphorylation of HMG-CoA reductase and thus decreased the activity of the enzyme. It is noteworthy that total activity was unaltered by the compounds. The unchanged total activity was consistent with the immunoblot analysis showing that the amounts of HMG-CoA reductase protein remained the same among the treatment and control groups.

Thiol-disulfide interchange also plays an essential role in modulating the activity of HMG-CoA reductase (16, 43). The susceptibility of HMG-CoA reductase to inactivation by sulfhydryl oxidation has been well documented (21, 22, 44, 45). The oxidative inactivation is reversible in the presence of high concentrations of thiols such as dithioerythritol, dithiothreitol and glutathione (21, 22) and may involve the formation of an intramolecular protein-SS-protein disulfide (44). In the present study, we used the lower DTT concentration (8 mmol/L) and pre-incubation of microsomes with phosphatase to test the possible effect on thiol redox status of HMG-CoA reductase by the garlic compounds. The total activity was not altered at 12 mmol/L DTT by the compounds. However, the enzyme activity of microsomes pre-incubated with phosphatase in the presence of 8 mmol/L DTT was decreased 10% by SAC. This finding is consistent with an early study demonstrating that the activity of HMG-CoA reductase was inactivated by diallyl disulfide derived from garlic (46). The inactivation was resulted from the formation of an intramolecular protein disulfide of the enzyme, indicating an increased sulfhydryl oxidation of HMG-CoA reductase by diallyl disulfide (46). However, unlike the finding of the present study, the inactivation of the enzyme by diallyl disulfide was irreversible by DTT. Although the reason for the difference between the two studies is not readily known, it is important to point out that for measurement of the enzyme activity, diallyl disulfide was added to isolated microsomes or
soluble 50 kDa HMG-CoA reductase, whereas SAC was incubated with hepatocytes in culture prior to the preparation of microsomes in the present study. Nonetheless, our data suggest that the decreased activity of HMG-CoA reductase caused by SAC also stems in part from sulfhydryl oxidation of the enzyme.

In summary, SAC, SEC, and SPC inhibit hepatic cholesterol biosynthesis primarily by decreasing HMG-CoA reductase activity. The decreased activity of HMG-CoA reductase by S-alk(en)yl cysteines, on the other hand, is attributable to increased phosphorylation but not gene expression of the enzyme. Among S-alk(en)yl cysteines, SAC appears to be the most potent inhibitor of HMG-CoA reductase, because it not only increases phosphorylation but also increases sulfhydryl oxidation of the enzyme.
5.6 REFERENCES


OVERVIEW

Intrigued by the hypolipidemic effects of garlic, the present studies using primary cultures of rat hepatocytes as a model were conducted to: i) identify the active constituents of garlic that inhibit cholesterol, TG and fatty acid syntheses; ii) determine the activities of lipogenic enzymes including FAS and G6PDH in order to elucidate the possible mechanisms on the reduction of TG synthesis by organosulfur compounds of garlic; and iii) elucidate the mechanism by which garlic compounds inhibit cholesterol synthesis.

To achieve these goals, four hypotheses were tested in the present studies. The first hypothesis was that the potency of organosulfur compounds of garlic in inhibiting cholesterol synthesis varied depending on chemical structure and solubility of the compounds. The results of the study showed that water-soluble organosulfur compounds of garlic including three S-alk(en)yl cysteines (SAC, SEC, and SPC), and three γ-glutamyl S-alk(en)yl cysteines (GSAC, GSMC, and GSPC), were active constituents responsible for the reduction of cholesterol synthesis. Among the water-soluble compounds tested, SAC, SEC, and SPC were more potent than GSAC, GSMC, and GSPC in inhibiting cholesterol synthesis, whereas alliin, SANC, SASA, and SMC had no effect on cholesterol synthesis. Although lipid-soluble compounds of garlic such as DADS, DATS, and DPDS appeared to abolish cholesterol synthesis, such inhibitory effect was attributed to cytotoxicity.

The second hypothesis was that the reduction of TG synthesis resulted from the inhibition of fatty acid synthesis due to decreasing the activity of lipogenic enzymes such as FAS and G6PDH. In fact, SAC, SEC, SPC, and GSMC inhibited hepatocyte fatty acid
synthesis. Among these compounds, SAC and SPC were more potent than SEC and GSMC on the inhibition of fatty acid synthesis. Furthermore, SAC and SPC not only decreased the activity of FAS but also inhibited TG synthesis. G6PDH, on the other hand, was not affected. Therefore, the results substantiate the notion that organosulfur compounds of garlic depress the activity of FAS leading to decreased fatty acid synthesis and TG production.

Thirdly, it was hypothesized that the inhibition on cholesterol synthesis by organosulfur compounds of garlic stemmed in part from the depressed activity of HMG-CoA reductase. Three S-alk(en)yl cysteines, i.e., SAC, SEC, and SPC, decreased cholesterol synthesis from acetate but not from mevalonate, suggesting HMG-CoA reductase as the point of regulation. This was further supported by the suppressed activity of HMG-CoA reductase by SAC, SEC, and SPC. Thus, sulfur compounds of garlic reduce hepatocyte cholesterol synthesis by depressing the activity of HMG-CoA reductase.

It was further hypothesized that the suppressed activity of HMG-CoA reductase resulted from decreased gene expression of the enzyme. Contrary to this hypothesis, the abundance of mRNA and protein concentration of HMG-CoA reductase were not altered by S-alk(en)yl cysteines suggesting that the compounds have no effect on gene expression of the enzyme. In order to further understand the possible regulatory mechanisms, the effect of the compounds on the phosphorylation and the thiol redox status of the enzyme was explored. The results showed that SAC, SEC, and SPC increased phosphorylation of HMG-CoA reductase and hence decreased the activity of the enzyme. In addition, SAC appeared to increase sulfhydryl oxidation of the enzyme. Taken together, the study suggests SAC may be more effective than other compounds in reducing cholesterol synthesis.
The current studies lead to a conclusion that water-soluble organosulfur compounds of garlic, especially SAC, SEC, and SPC, are the most important compounds responsible for the lipid-lowering effect of garlic, which in turn may reduce the risk of CVD. The cause of decreased TG synthesis is explained in part by the inhibition of fatty acid synthesis by depressing FAS. The inhibition of hepatic cholesterol biosynthesis by S-alk(en)yl cysteines, on the other hand, stems primarily from decreased HMG-CoA reductase activity. The mechanisms by which S-alk(en)yl cysteines depress HMG-CoA reductase activity is attributed in part to phosphorylation but not gene expression of the enzyme. In addition to phosphorylation of HMG-CoA reductase, SAC appears to increase sulfhydryl oxidation of the enzyme and hence further decrease the enzyme activity. Clearly, organosulfur compounds of garlic modify the activity of HMG-CoA reductase at the posttranslational but not pretranslational level.

More importantly, the present studies have added to our understanding of the active constituents responsible for the lipid-lowering effect of garlic and the mechanisms of garlic action in regulating cholesterol and triglyceride synthesis. The results also provide impetus for potential pharmaceutical use of garlic compounds in the prevention and treatment of CVD.

Whether garlic compounds regulate the expression of LDL receptors warrant further study. In addition, future in vivo studies including animal and human studies are needed to confirm the findings from our in vitro studies.
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Yeh, Y.Y., and Liu, L. (2001) Cholesterol-lowering effect of garlic extracts and organosulfur
Liu, L., and Yeh, Y.-Y. (2000) Inhibition of cholesterol biosynthesis by organosulfur
   compounds derived from garlic. Lipids 35: 197-203.